IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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First Named Inventor	: Jon ADLER			
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Title	: T2r Taste Receptors and Genes Encoding Same			

APPEAL BRIEF

Mail Stop Appeal Brief- Patents
Commissioner for Patents
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Sir:

On May 14, 2004, Appellants appealed to the Board of Patent Appeals from the final rejection of claims138-157. The following is Appellants' Appeal Brief submitted pursuant to 37 C.F.R. § 1.192.

Real Party in Interest

The real party in interest is Senomyx Incorporated, a corporation located in La Jolla, California and incorporated in Delaware.

Related Appeals or Interferences

There are no related appeals or interferences known to Appellants.

Status of Claims

Claims 158-185 remain pending. All of these claims stand finally rejected and are appealed herein.

Status of Amendment

An amendment pursuant to 37 C.F.R. § 1.116 canceling then-pending claims 138-157 in favor of new claims 158-185 was submitted on May 14, 2004.

This amendment was entered in its entirety (See Advisory Action dated June 9, 2004). The Examiner finally rejected these claims under 35 U.S.C. § 101 and § 112 first paragraph (See the Advisory action mailed on June 9, 2004).

Summary of Claimed Subject Matter

Independent Claim 158 is directed to an isolated nucleic acid molecule encoding a bitter taste receptor polypeptide selected from the following:

(i) the nucleic acid sequence contained in SEQ ID NO: 7;[1]

(ii) a nucleic acid sequence which encodes a bitter taste receptor polypeptide having the polypeptide sequence contained in SEQ ID NO: 8;

(iii) an isolated DNA that hybridizes under high stringency hybridization conditions to the nucleic acid sequence contained in SEQ ID NO: 7 [precise hybridization conditions recited in claim 158].

As disclosed in the subject application, the nucleic acid sequences in claim 158 correspond to a human taste receptor polypeptide which is a member of the T2R (bitter taste receptor) family and related sequences which receptor is referred to in the application as "hT2R61" or "T2R61". Specific support for this claim may be found at least in the Sequence Listing, page 71, lines 21 through 46; the disclosure at pages 4-20 wherein the specification describes the identifying characteristics of T2R bitter taste receptors [T2R61 is a member of the T2R gene family], original claim 1, and the disclosure at page 30, line 31 to page 31, line 27.

Independent Claim 159 relates to an isolated nucleic acid molecule encoding a bitter taste receptor (T2R) polypeptide having at least 95% sequence identity to the polypeptide contained in SEQ ID NO: 8 (hT2R61 polypeptide). Specific support for this claim may be found at least in the SEQUENCE

LISTING, page 71, lines 21-46, original claim 9, and the disclosure at pages 4-20 (wherein the specification describes the identifying characteristics of bitter taste receptors which are members of the T2R family). (Therefore, this independent claim on Appeal similarly relates to a nucleic acid sequence encoding a novel member of the T2R family, human T2R61, and nucleic acid sequences encoding bitter taste receptor polypeptides which are at least 95% identical thereto).

Independent Claim 160 is directed to a nucleic acid sequence encoding a bitter taste receptor consisting of the nucleic acid sequence contained in SEQ ID NO: 7 (hT2R61 nucleic acid sequence). Specific support for this claim may be found e.g., in the SEQUENCE LISTING, page 71, lines 21-46, original claim 1, clause (i), and the Specification at pages 4-20 wherein the identifying characteristics of T2R gene members are generally described.

Independent Claim 161 is directed to a nucleic acid sequence encoding the bitter taste receptor polypeptide contained in SEQ ID NO: 8 (hT2R61 polypeptide) operably linked to a promoter sequence. Specific support for claim 161 may be found at least in the SEQUENCE LISTING page 71, lines 21-46, original claims 1, 36 and 37, and the Specification at pages 4-20 wherein the identifying characteristics of T2R bitter taste receptor members are generally described.

Dependent Claims 162-170 similarly relate to isolated nucleic acid sequences which encode a bitter receptor polypeptide having the amino acid sequence contained in SEQ ID NO: 8 (hT2R61 polypeptide); nucleic acid sequences which hybridize thereto under defined stringent hybridization conditions; or nucleic acid sequences which encode T2R polypeptides which possess at least 95% sequence identity to the hT2R1 polypeptide contained in SEQ ID NO: 8. These claims further provide for such nucleic acid sequences to

1 The nucleic acid and polypeptide sequences contained in SEQ ID NO: 7 and 8 respectively encode or contain the amino acid sequence of a taste receptor polypeptide referred to as T2R61 in the subject application.

be operably linked to a promoter sequence that regulates the transcription thereof or a chaperone sequence that facilitates secretion thereof. The remaining dependent claims are directed to expression vectors containing such nucleic acid sequences (appealed claims 172-173), or cells which are transfected or transformed by such isolated nucleic acid sequences (appealed claims 174-185).

All of the Appealed claims require as an essential element hT2R61 nucleic acid sequences which encode a member of the T2R taste receptor family heretofore unknown. As disclosed in the Specification, T2Rs constitute a family of G protein coupled receptors (GPCRs) that are involved in taste transduction (See, for example page 4, lines 1-10), particularly bitter taste transduction. Prior to the present invention, it had been recognized that a family of taste receptor genes (T2Rs) expressed at least in rodents and humans, and most probably other mammals, existed and was implicated in the control of bitter taste (See page 11, lines 9-28 of the Specification). Also, prior to the present invention, functional data had been reported in the literature substantiating that T2Rs are specifically activated by bitter ligands. For example, it was reported that a mouse member of the T2R family, mT2R5, was responsive to cycloheximide (bitter ligand), and that some mutations in this gene disrupted the cycloheximide taste phenotype. Additionally, it had been reported that a human T2R (hT2R-8) and mouse T2R-4 respectively specifically respond to the bitter ligands denatonium and PROP.

The inventors identified the subject hT2R61 as a member of the T2R bitter taste receptor family based on various structural, genetic, tissue expression profiles and other information that is consistent with other T2R members. The shared characteristics of T2Rs which are disclosed in this Application include the following:

(i) Human T2R genes are comprised in three defined gene clusters, which together comprises about 25 genes: gene interval 12p13 corresponds to a gene locus found on mouse chromosome 6 previously implicated in the regulation of taste perception to bitter ligands including, e.g., sucrose octaacetate, raffinose,

undecaacetate cycloheximide and quinine (Lush et al., *Genet. Rev.* 6:167-74 (1995)) reference at (Specification, page 11, lines 8-17); gene interval 5p15, a gene locus previously correlated to the ability to taste PROP (bitter ligand). (Reed et al. *Am J. Hum, Genet.* 64:1478-80 (1979)) (Specification page 11, lines 9-17), and the 7q33 gene cluster (Specification, page 11, lines 1-8).

(ii) All T2Rs identified to date have a somewhat conserved structure which exhibit a requisite degree of nucleotide sequence identity to other T2R members (Specification, page 11, line 21 to page 12 line 6), and comprise conserved sequences motifs. (Specification, page 11, line 21 to page 12, line 25).

(iii) All T2Rs are selectively expressed in subsets of taste receptor cells comprised in the tongue, palate epithelium, geschmackstreiffin and epiglottis (page 12, lines 7-9) and are less expressed by fungiform papillae (page 12, line 10); and

(iv) All T2Rs are selectively expressed in gustducin-positive cells. Because the subject T2R61 gene possesses properties characteristic of other T2R genes, it was identified to be a member of the T2R family. Based on the identification of hT2R61 as a member of the T2R gene family, the subject specification discloses that hT2R61 nucleic acid sequences have various applications, *e.g.*, they may be used in screening assays conventionally used to identify modulators of G protein coupled receptors (See general disclosure at pages 45 to 67).

The subject Application specifically teaches e.g., at pages 54-56 that the subject T2R nucleic acid sequences have practical applications including e.g., (i) the genetic detection of subjects having mutations that impair bitter taste ligands (ii) probes for use in constructing taste topographic maps; (iii) probes for identifying bitter taste cells; and (iv) most significantly, the identification of ligands that modulate (agonize, antagonize or enhance) bitter taste transduction elicited hT2R61. [These ligands are disclosed to possess potential application as

food ingredients, for example in blocking or masking the bitter taste of bitter compounds which trigger hT2R61-associated bitter taste.]

Grounds of Rejection

§ 101 Rejection

All of appealed claims 158-185 stand rejected under 35 U.S.C. § 101, on the basis that the claimed invention, which relates to hT2R61 nucleic acid sequences, and cells or vectors containing, is not supported by "either a specific and substantial asserted utility or a well established utility" (See page 2, lines 13-15 of Advisory Action mailed June 9, 2004).

While other T2R ge

nes were demonstrated to encode functional bitter taste receptors at the time the application was filed, the Examiner maintains that "it was impossible to predict that a particular T2R would bind a bitter tastant." (Advisory Action page 5, lines 12-13). The Examiner further maintains that while the Specification correctly teaches that hT2R61 is a bitter taste receptor, that the claims lack a practical utility because the Specification "says nothing about SEQ ID NO: 8 binding to [a particular bitter ligand] nitrosaccharin, and the Examiner can think of no way to use SEQ ID NO: 8 in a way that constitutes a substantial utility without this knowledge". (Advisory Action, page 2, lines 18-20).

Also, the Examiner maintains that the claims which embrace variants of the exemplified hT2R61 nucleic acid sequence (SEQ ID NO: 7) lack practical utility absent a demonstration that they encode functional bitter taste receptor polypeptides.

§ 112 First Paragraph Enablement Rejection

All of claims 158-185 were rejected under 35 U.S.C. § 112 first paragraph, on the basis that one skilled in the art would not "know how to use the claimed invention so that it would operate as intended without undue experimentation."

In the § 112 first paragraph enablement rejection of claims 158-185, the Examiner essentially incorporates the same reasoning as the § 101 rejection. The Examiner states as follows:

> Specifically, since the claimed invention is not supported by a substantial asserted utility, for the reasons set forth above, one skilled in the art would not know how to use the claimed invention so that it would operate as intended without undue experimentation.

With particular respect to the hT2R61 gene variants encompassed by the Claims 158-159 and 169-185, the Examiner further states as follows:

> Applicant has not provided sufficient guidance as to how to make and use the encoded polypeptides which are not 100% identical to the polypeptide of SEQ ID NO: 8, but which retain a desired property of the polypeptide of SEQ ID NO: 8.

Written Description Rejection

Claims 158, 159, 164-185 were also rejected under 35 U.S.C. § 112 first paragraph based on lack of written description. Particularly, the Examiner states the following:

> [The claims on appeal]
>
> contain[ing] subject matter which was not described in the specification in such a way to reasonably convey to one skilled in the relevant art that the inventor(s), at the time of the application was filed, had possession of the claimed invention.

The Examiner alleges that the Specification does not establish that Appellants were in possession of the claimed invention relating to isolated nucleic acid sequences which encode a bitter taste receptor as set forth in claims 158-159 and 164-185. The basis of the rejection is that it was allegedly unpredictable how to select specific nucleic acid sequences according to the claims that encode functional bitter taste receptors.

For example, the Examiner states:

> [T]he Specification has not provided a particular essential feature, either a functional or structural feature, that the claimed genus of polynucleotides possess. The recitation of the property of hybridization does not, alone, provide sufficient information regarding the structure of the claimed polynucleotide variants." (Office Action dated November 14, 2003, page 14, last paragraph).

Also, the Examiner states in the same Office Action the following:

> Similarly, the recitation of a percent identity to SEQ ID NO: 8 provides no description of any amino acid sequence other than that of SEQ ID NO: 8. The specification has not defined what particular common structural or functional properties are possessed by the claimed genus of polynucleotides.

For reasons set forth *infra,* Appellants respectfully submit that each of these rejections is unsustainable as all of the claims satisfy the § 101 utility, § 112 enablement and § 112 written description guidelines.

It will be shown:

(i) that the Specification does identify at least one credible practical utility for the claimed isolated nucleic acid sequence which encode bitter taste receptors polypeptides;

(ii) that the Specification does enable how to make and use the claimed nucleic acid sequences which encode bitter taste receptors in assays to identify (bitter) taste modulatory compounds; and

(iii) that the Specification does establish that Applicants were in possession of the claimed isolated nucleic acid sequences which encode bitter taste receptor polypeptides.

Arguments

Arguments in Traversal of § 101 Rejection of Claims 158-185

Appellants respectfully maintain that the as-filed Specification provides sufficient information for one skilled in the art to reasonably conclude that the isolated nucleic acid sequences recited in each of independent claims 158-161 encode a human gene (referred to hereinafter as hT2R61) or a functional variant thereof which affects bitter taste transduction. It further would have been routine and apparent to one skilled in the art, based on the as-filed disclosure, to have used the subject nucleic acid sequences in any of the various GPCR screening assays which are described in the subject specification to identify bitter ligands which specifically activate or modulate the activity of the hT2R61 receptor and therefore modulate hT2R61-mediated bitter taste transduction.

Restatement of Examiner's Basis for the § 101 Rejection

The position of the Examiner is that the Specification does not contain sufficient information for one skilled in art to have reasonably concluded that the claimed isolated hT2R61 nucleic acid sequences according to Claims 158-185 encode a functional member of the T2R taste receptor family which is involved in bitter taste transduction. Appellants respectfully disagree.

As discussed *supra,* the present invention relates to the identification of a novel G-protein coupled receptor referred by Applicants in the Specification as

hT2R61. The Specification discloses that hT2R61 is a taste cell specific GPCR which is a component of the taste signal transduction pathway, particularly the bitter taste signal transduction pathway, which mediates bitter taste when it associates with a G-protein, e.g., gustducin. (See the Specification at page 8, lines 5-28, et seq.) The Specification specifically teaches that the hT2R61 nucleic acid sequence encode a previously unidentified member of the T2R taste receptor family.

Because hT2R61 was reasonably identified to be a member of the T2R taste receptor family, the Specification indicates that the hT2R61 nucleic acid sequences and variants thereof [as claimed herein] possess practical utility, for example, as probes to detect taste cells, for use in constructing genetic taste topographic maps, as probes to detect mutations associated with taste-associated deficiencies, and in screening assays to detect compounds that modulate bitter taste signaling (which compounds can be used as additives for foods, pharmaceuticals and the like). (See e.g., the Specification at page 14, line 1 to page 15, line 24 et seq.)

During prosecution, the Examiner took the position that the hT2R61 nucleic acid sequence lacks a substantial or well-established utility essentially for two reasons:

(1) it was unpredictable that the disclosed hT2R61 polynucleotide sequence or variants thereof encoded a bitter taste receptor; *i.e.,* a functional T2R, and

(2) the exemplified hT2R61 nucleotide sequence and variants thereof, according to the claims on Appeal , even assuming they were known to encode a bitter taste receptor, lack utility absent the recognition and disclosure by the inventors, in their as-filed their patent application, that this receptor interacts specifically with nitrosaccharin. Appellants respectfully disagree.

The Specification Identifies A number of Properties Which Are Shared by Different T2R Members Which Would Have Reasonably Suggested That hT2R61 Encodes a Bitter Taste Receptor Absent Functional Data

As of the date of invention, the existence of a family of GPCRs expressed in taste cells, which are associated with bitter taste detection, was known in the art. This family of genes, T2Rs, were further known to share various structural, genetic, expression, and functional characteristics. Based on these identifying criteria, the inventors reasonably identified hT2R61 to be a member of the T2R gene family which is involved in bitter taste.

In particular, the Specification discloses that T2R genes, at the time of invention, were known to comprise a family of related seven transmembrane G protein coupled receptors, which do not contain introns, that interact with a G protein [e.g., gustducin] to mediate taste signal transduction (Page 11, lines 23-28 of the Specification). The subject hT2R61 nucleic acid sequences possess a 7-transmembrane structure characteristic of a GPCR and do not possess introns.

Additionally, the Specification discloses that the nucleotide sequence of T2R members were known to share on average about 20-30% identity over a region of at least about 50 nucleotides in length relative to other members and to comprise regions that encode consensus amino acid sequences or domains that are characteristic of and which are shared by all T2R family members. (These consensus sequences are provided at page 12, lines 21-32 of the subject Specification). hT2R61 possesses such consensus sequences and the requisite percentage of sequence identity to other T2Rs. In fact, because of this sequence identity to other T2Rs, hT2R61 was isolated using to another human T2R gene, hT2R1, as a probe.

Still further, the Specification discloses that T2Rs were known to be selectively expressed in specific subsets of taste receptor cells of the tongue,

palate epithelium, foliate geschmackstreifen and epiglottis, and are less expressed in fungiform papillae. T2R61 meets this criterion.

Yet additionally, the Specification teaches that T2Rs are selectively expressed in gustducin-positive cells (See page 12, lines 6-10 of the Specification). T2R61 meets this criterion.

Additionally, the Specification discloses that human T2R members are comprised in clusters in three human gene loci that have been shown in humans and rodents to correlate to the regulation of bitter taste. The first human gene loci, 5p15, is a locus previously correlated to the ability to taste PROP, a bitter ligand (Reed et al., *Am. J. Human Genet* 69:1478-80 (1999) The second human gene loci, 12p13, corresponds to a region of mouse chromosome 6 that contains a number of genes that were reported to influence the taste perception of bitter ligands such as sucrose octaacetate, raffinose, undecaacetate cycloheximide and quinine (Specification at page 11, lines 1-22). The third human gene loci 7q33 comprises about 7 T2R genes. hT2R61 is found in the 12p13 gene locus.

Yet additionally, the Specification teaches that T2R genes meeting these criteria have been found to encode taste receptors which specifically respond to bitter ligands. Particularly, the Specification teaches at page 11, lines 11-22 that mT2R-5 specifically responds to cycloheximide, and that mutations in the gene have been correlated to an inability to taste cycloheximide. Further, the Specification teaches that mT2R-4 responds to bitter ligands denatonium and PROP as does the human T2R-8 gene. Thus, functional data was available substantiating the reasonable expectation that T2R genes encode functional bitter taste receptors.

Based at least on this plurality of different criterion, the subject inventors <u>reasonably</u> concluded that the hT2R61 nucleic acid sequence encodes a functional member of the T2R gene family which responds to bitter taste ligands and is involved in bitter taste transduction.

Notwithstanding all of this information, the Examiner concluded that it could not be reasonably predicted that hT2R61 encodes a functional taste receptor absent functional data. However, Appellants respectfully submit that no evidence or convincing reasoning has been set forth which would raise significant doubt regarding the anticipated functionality of the claimed hT2R61 nucleic acid sequences. (Indeed, as discussed *infra,* functional data provided during prosecution has confirmed that the hT2R61 nucleic acid sequence (SEQ ID NO: 7) does encode a functional bitter taste receptor as disclosed in the subject application.)

The Examiner has not pointed to a single T2R gene isolated from the human or rodent genome that possesses the identifying characteristics of a T2R member which is not functional. Rather the Examiner concludes without reasonable basis or evidence that because only a few members of the T2R family have been functionalized (proven to bind to specific bitter ligands) that this establishes the unpredictability of the outcome.

However, the absence of functional data relating to other T2Rs does not support a conclusion that other T2R genes do not encode functional bitter taste receptors. More to the point, it does not support a conclusion that it was unpredictable that the claimed hT2R61 nucleic acid sequence encodes a bitter taste receptor.

This conclusion ignores the fact that those skilled in the art <u>irrespective of the fact that functional data was available for only a few T2R gene members</u>, classified a group of genes possessing the identifying characteristics of hT2R61 as T2Rs, *i.e.,* GPCRs which are involved in bitter taste transduction. This fact is evidenced by at least two publications incorporated by reference in this application, Chandrashekar et al., *Cell,* 100:700-11 (2000) and Adler *Cell* 100:693-702 (2000), references considered during prosecution of this application. (Exhibit 1 and Exhibit 2)

See, for example, Figure 1 of Adler et al which aligns different human, rat and mouse T2R genes, and the authors' statement at page 695 that they "presume that the high degree of variability between T2Rs reflects that need to recognize many structurally different ligands." This statement indicates that those skilled in the relevant art, who were aware of the number and diversity of different T2Rs, reasonably expected the various members to be functional and to bind different bitter ligands.

Thus, in contrast to the position of the Examiner, the expectation of the skilled artisan at the time of invention was and remains that nucleic acid sequences which possess the criteria identified above are properly classified as T2Rs and encode polypeptides involved in bitter taste transduction.

In further support of Appellants' argument that inventors <u>reasonably</u> and <u>correctly</u> disclosed that hT2R61 is a bitter taste receptor, Appellants submitted experimental data with the Reply dated August 21, 2003 substantiating that hT2R61 interacted with a known bitter ligand (nitrosaccharin). In addition, when hT1R61 was screened against a library of about 15,000 compounds of unknown taste properties, it interacted with compounds in the 3,4-dihydro-isoquinoulin-1-one class [(-4-(4-benzo[1,3]dioxol-5-ylmethyl-piperazine-1-carbonyl)-3-(4-methoxy-phenlyl)-2-methyl-3,4-dihydro-2H-isoquinolin-1-one and -3-(4-methoxy-phenyl)-2-methyl-4-(4-phenyl-piperazine-1-carbenyl)-3,4-dihydro-2H-isoquinolin-1-one) as well as three compounds in the benzothiazole class ([2-(PROP-2-ene-1-sulfonylsoulfenyl)-benzothiazole, -2-[2-(4,6-Dimethyl-pyrimidin-2-ylsulfanyl]-ethanesulfonyl]-benzothiazole and 1,2-bis (sulfonylbenzo-thiazole)ethane). As anticipated, when these compounds were evaluated in human taste tests, they were found to elicit a bitter taste. (Exhibit 3). Thus, Appellants submitted convincing functional data during prosecution substantiating that the subject hT2R61 polynucleotide encodes a bitter taste receptor that responds to a known bitter compound (nitrosaccharin) and other bitter compounds.

These additional experimental data were considered by the Examiner (See page 11, ¶ 1 of Office Action dated November 11, 2003) but was found to be unpersuasive allegedly because "the specification has given no indication as to which of these compounds is expected to bind to and activate SEQ ID NO: 8 [hT2R61]." Absent such information, the Examiner alleged that the Specification merely provides a "plan of research" and that "each of the proposed uses are of a general nature, and it would require undue experimentation on the part of the skilled artisan to determine what, particularly, the claimed polynucleotides could be used for." (November 11, 2003 Office Action, page 7, lines 10-12). Appellants again respectfully disagree.

As noted above, high throughput assays conducted with about 15,000 thousand compounds, identified 5 compounds which specifically activated the hT2R61 receptor, all of which were confirmed in human taste tests to be bitter compounds. These results <u>confirm</u> that hT2R61 is a bitter taste receptor and further confirm that this receptor can <u>predictably</u> be used in high throughput assays to identify taste modulatory compounds.

Appellants further respectively submit that it was reasonable for the inventors to have concluded that the nucleic acid sequence identified as hT2R61 (SEQ ID NO: 7) encodes a functional bitter taste receptor based on publicly available functional data relating to other T2R family members contained in two peer-reviewed references which are incorporated by reference in their entireties in the subject application. (Adler et al, *Cell*, 100:693-702 (2000); Chandrashekar et al., *Cell*, 100:700-11 (2000) (Exhibit 1 and 2) (Both references are incorporated by reference in their entireties at page 8, lines 12-13 of the Specification and were considered by the Examiner during prosecution of this application). The Chandrashekar (*Id.*) reference contains functional data which establishes that several previously identified T2R member (mouse T2R-5, mouse T2R8 and hT2R-4), when tested in HEK-293 cells (which express a single T2R and a G protein) with a small battery of compounds (55) (which included bitter and sweet

tastants, *i.e.,* various amino acids, peptides, and other natural and synthetic compounds) responded to several bitter ligands. Appellants respectfully submit that this functional data supports a conclusion that it would have been reasonably predictable that nucleic acid sequences encoding other T2R members, such as the T2R61 sequences claimed herein, would likewise encode functional bitter taste receptors which specifically respond to bitter taste ligands.

Additionally, the Adler (*Id.)* reference incorporated by reference, further teaches that while it was known that there existed a plurality of different rodent and human T2R members which exhibit substantial sequence identity to one another, that these T2Rs contain regions of hypervariability which were postulated to facilitate the interaction of different T2Rs with structurally dissimilar bitter ligands. (*See* pages 697-699 of Exhibit 2).

Particularly, Adler states the following: "Our finding that each taste receptor cell expresses a large number of T2Rs is consistent with the observation that mammals are capable of recognizing a wide range of bitter substances, but not distinguishing between them". (Page 700, right hand column, Exhibit 2).

Therefore, the prevailing view of experts in the field at the time of the invention was that while T2Rs comprise a fairly large family of taste receptors that regulate bitter taste transduction, that this large number of T2R genes are functional and recognize structurally distinct bitter ligands.

As noted above, the Examiner considered the later-obtained experimental data confirming that hT2R61 is functional (Exhibit 3) and other information in the Chandrashekar and Adler references but did not find it to be persuasive to overcome the § 101 rejection. For example, the Examiner stated in the Advisory Action that: "it is agreed that Chandrashekar and Adler provide the evidence that certain T2R members are, in fact, bitter taste receptors. However, the state of the art, exemplified by Chandrashekar and Adler, indicates that it was impossible to predict that a particular T2R would bind a bitter tastant." The

Examiner relies on the fact that Chandrashekar tested 25 murine T2Rs and 11 human T2Rs and "only" identified two mouse T2Rs (mT24-5 and mT2R-8) and one human T2R (hT2R-8) that specifically responded to bitter taste ligands. This was purported to support a conclusion that it is unpredictable whether a given T2R is functional.

Appellants respectfully disagree with this assessment of the Chandrashekar and Adler references. The experiments described in these references only screened T2R expressing cells with a small number (55) of compounds (which included for example sweet and bitter ligands) Therefore, it is not unexpected, given the small number of compounds screened, that only several of these T2Rs specifically interacted with a bitter compound contained in this compound sample. Indeed, not all of the screened compounds were even bitter ligands.

The Examiner's conclusion is further unsupportable because it ignores the statement by Adler (Exhibit 2) that "the high degree of variability between T2Rs reflects the need to recognize many structurally diverse ligands" (See page 65, left hand column, last five lines). Based thereon, the <u>reasonable</u> conclusion, more consistent with the teachings of Adler and Chandrashekar, is that most or all of the disclosed full length T2R genes disclosed therein encode functional bitter taste receptors and this would have been demonstrated by screening these T2R polypeptides against a more structurally diverse and larger compound library.

High throughput screens <u>routinely</u> effected to identify ligands that interact with GPCRs conventionally screen a GPCR against compound libraries containing hundreds or even thousands of compounds. This is routine given the wide commercial availability of many large and structurally diverse compound libraries.

In further rebuttal of the utility rejection of claims 157-185, Appellants submitted a Declaration by Dr. Mark Zoller, an expert in the field of GCPRs and assays used for the identification of GPCR ligands, and taste GCPRs specifically. (Exhibit 4). This Declaration was considered but also did not convince the Examiner that the utility and other rejections are unsupportable.

In this Declaration, Dr. Zoller avers that in his expert opinion, the available information relating to hT2R61 which existed at the time of invention would be sufficient for a skilled artisan to reasonably conclude that the claimed hT2R61 nucleic acid sequence encodes a functional bitter taste receptor. For example, Dr. Zoller notes for example that the gene is present at a loci in the human genome correlated with bitter taste, it specifically hybridizes to another human T2R (T2R1), it possesses a sequence which exhibits the closest homology to other T2Rs; it possesses a domain structure consistent with its encoding a GPCR; it is expressed in a subset of taste cells characteristic of other T2R members (which differs from sweet and umami taste receptors), and finally consistent with other T2R members it is expressed in association with the G protein gustducin. (*See,* ¶ 5-10 of Zoller Declaration)

This information, coupled with the prior knowledge that related T2Rs encode functional bitter taste receptors, (Zoller Declaration, ¶11) would reasonably suggest that hT2R61 would possess a similar function. Dr. Zoller further opines that there was a consensus in the relevant scientific community at the time of invention that T2R genes as a whole encode functional bitter taste receptors. [Dr. Zoller explains that the location and organization of T2Rs in the human genome is consistent with gene duplication events leading to gene expression and the functionality of the various T2R members as bitter taste receptors]. (See ¶ 12 of Zoller Declaration)

Also, Dr. Zoller avers that later- experimental results obtained by the inventors have confirmed their expectations, *i.e.,* hT2R61, has been shown to specifically bind to bitter ligands (*e.g.*, nitrosaccharin). Moreover, Dr. Zoller

notes that the functionality of hT2R61 has also been confirmed by a second experimental group (who showed that hT2R61 binds saccharin). (Zoller Declaration, ¶ 13)

Notwithstanding the overwhelming and convincing evidence contained in the Zoller Declaration, the Examiner has maintained his position that it was "unpredictable" that hT2R61 could have been shown to be functional and alleges that the Specification merely provides an "invitation to experiment" with no likelihood of a successful outcome. The Examiner again relies on the Chandrasekhar and Adler references which tested 36 T2Rs with 55 compounds and presented functional data showing that 3 of these T2Rs specifically responded to bitter ligands. The Examiner suggests that these results provide evidence that the functionality of T2R members was unpredictable at the time of invention

Appellants respectfully disagree with the Examiner's conclusion. Appellants again emphasize that the experiments reported in these references only screened the tested T2Rs with a very small number of compounds (55), and not all of these were even bitter ligands. By contrast, when Appellants screened T2R61 against about 15 thousand compounds (as noted above, the use of large compound libraries is routine in the GPCR assay field) Appellants obtained 5 positive hits (Exhibit 3), <u>all of which were shown to elicit a bitter taste in human taste tests</u>. This result, which has not been refuted by the Examiner, confirms the <u>predictability</u> of identifying T2R modulators under more typical assay conditions (*i.e.,* greater number of screened compounds of different structural classes).

The Examiner further indicated that the Zoller affidavit (as well as earlier submitted functional data which showed that hT2R61 is specifically activated by the bitter ligand nitrosaccharin and other bitter compounds) is insufficient to overcome § 101 utility rejection because "The Examiner can think of no assay that could be used to block the specific activation of hT2R61 without using

nitrosaccharin. Conversely, the invitation to perform extensive investigation to try to find a ligand that activates hT2R61 does not constitute a substantial utility.". Appellants respectfully disagree.

Contrary to the Office Action, the practical utility of hT2R61 does <u>not</u> require that it be used in conjunction with nitrosaccharin nor does the identification of bitter ligands that interact with hT2R61 require extensive investigation. Rather, as the Specification [correctly] indicates, the hT2R61 sequence encodes a bitter taste receptor, and therefore be used as a probe in genetic screening assays to identify subjects who comprise mutations in the gene and who are potentially at risk for impaired ability to taste some bitter ligands. In support of this utility, the Specification at page 11, lines 9-19 indicates that mutations in other T2R genes have been shown to correlate to the inability to taste cycloheximide. Also, the Specification teaches at page 10, lines 24-25 that "the [T2R] nucleic acids and [T2R] polypeptides they encode can be used as probes to dissect taste-induced behaviors," as well as "investigation of taste transduction regulation and specific investigation of taste receptor cells."

However, most significantly, and as correctly disclosed in the as-filed application, the subject T2R nucleic acid sequences, based on their reasonably anticipated [and later proven] functionality as bitter taste receptors can be used in high throughput screens to identity compounds which modulate the activity of this receptor. Contrary to the Advisory Action, this is a substantial utility because it was reasonable to conclude that the claimed hT2R61 sequence encoded a T2R which would be shown to bind to bitter ligands. This is to be contrasted with the typical "orphan receptor" rejection scenario wherein an Applicant identifies and claims a novel gene but is uncertain of its in vivo functionality. By contrast, Applicants are claiming a gene <u>which is a member of a family of GPCR's having an established function accepted by experts in the relevant field at the time of the invention</u>, namely they encoded GPCRs involved in bitter taste transduction. This fact is substantiated by at least the Adler and

Chandrashekar publications (Exhibits 1 and 2) which describe the various characteristics of T2R genes as a whole (*See* Zoller Declaration). The fact that T2R61 possesses properties characteristic of other T2Rs (Zoller Declaration, ¶¶ 5-10) characteristics, coupled with the demonstrated functionality of other T2R members, is sufficient to reasonably suggest that the claimed hT2R61 nucleic acid sequence encoded a human taste receptor that affects [along with other human T2R members] bitter taste transduction.

In contrast to the Advisory Action, it was not "impossible to predict" that hT2R61 would bind a bitter tastant. Rather, it was reasonably predictable that high throughput screens effected with a sufficient number of bitter ligands would have identified bitter ligands which activate hTR61. Appellants' reasonable expectation has been proven to be correct.

The fact that it was reasonable for the inventors to anticipate that the claimed hT2R61 nucleic acid sequences encode a bitter taste receptor which could be used to identify bitter ligands which activate this receptor is further substantiated by the fact that another research group, who subsequently identified the hT2R61 sequence (as well as other human T2Rs discussed in this application) similarly reported that the T2R61 sequence (as well as other T2Rs) encoded bitter taste receptors absent supporting functional data (See ¶ 12 of Zoller Affidavit).

Still further evidence that the subject application provides a substantial utility for hT2R61 is the fact that saccharin was among the 55 compounds screened by Chandrashekar et al. (Exhibit 1) as being a potential T2R ligand. The Examiner considered this argument but indicated that it was not "clear that Chandrashekar used saccharin as a potential bitter tastant, rather it appears that Chandrashekar used it as a sweet tastant, *i.e.,* the concentration was 10mM and is not stated by Chandrashekar to be high as to be perceived as bitter." Appellants respectfully disagree. The Chandrashekar reference provides

compelling evidence that saccharin was considered to potentially be a T2R ligand.

Thus, the teachings of Chandrashekar would reasonably suggest to a skilled artisan that saccharin desirably should be included in compound libraries to be screened for potential hT2R61 modulators, especially given the fact that this compound was notoriously well-known to elicit an undesirable bitter after-taste in many human subjects.

For similar reasons, the Examiner further suggests that the Specification does not reasonably establish that the inventors established the functionality of the genus of nucleic acid sequences encompassed by the claims on Appeal. The Examiner has suggested that one skilled in the art "would not be able to make useful predictions as to the nucleotide positions or identities of those sequences based on the information in the specification."

Also, the Examiner states that the "instant disclosure of a single polypeptide, that of SEQ ID NO: 7, encoding a polypeptide with no instantly disclosed specific activities, does not adequately support the scope of the claimed genus." Appellants respectfully disagree.

At the outset, Appellants note that the only independent claims which encompass hT2R61 sequence variants (Claims 158-159) respectively require:

(1) that the claimed isolated nucleic acid sequence specifically hybridize to SEQ ID NO: 7 under high stringency conditions and encode a functional bitter taste receptor (Claim 158 and Claims dependent thereon); or

(2) that the nucleic acid encode a polypeptide that is at least 95% identical to the native hT2R61 polypeptide (Claim 159 and claims dependent thereon).

Based on the teachings of the Specification and claims one skilled in the art would readily be able to select nucleic acid sequences that hybridize to

hT2R61 or to synthesize nucleic acid sequences which encode a T2R polypeptide that is at least 95% identical to hT2R61 (SEQ ID NO 8). In contrast to the final rejection, the genus of sequences encompassed by the claims is hardly "essentially limitless".

For the reasons set forth above the Specification provides substantial information relating to assays that had been used previously to identify bitter ligands which specifically interact with T2R polypeptides.

One skilled in the art, therefore, would understand from the teachings of this application how to screen variant hT2R61 sequences according to the claimed invention to assess whether such variants encode T2R polypeptides that retain the desired function (specifically interact with hT2R61-specific bitter ligands). Given the anticipated high degree of sequence identity of polypeptides encoded by the claimed nucleic acid sequences to endogenous T2R61, it would be reasonably predictable that a substantial number of these variant T2R61 nucleic acid sequences would encode T2R61 variants that are likewise functional. This functionality would be shown using T2R assays disclosed in the specification which would identify those T2R variants which retain the functionality of hT2R61 (SEQ ID NO 8) (i.e., bind the same bitter ligands as hT2R61 polypeptide).

As native hT2R61 would be anticipated to be (and in fact has been proven to be) functional, it similarly would have been reasonably predictable that many T2R61 variants encoded by the claimed nucleic acid sequences would be functional as well.

Therefore, based on the evidence of record, the utility rejection should be vacated because the functionality of hT2R61 as a bitter taste receptor and variants thereof as claimed herein would be credible to experts in the field based on the evidence of record.

According to MPEP § 2107, an Examiner should review the claims and the supporting written description to determine whether the utility requirement has been satisfied. No rejection based on lack of utility should be made, if an applicant has asserted a specific and substantial utility that would be considered credible by one of ordinary skill in the art.

In most cases, an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. § 101, MPEP 207.02 III A. The Court of Customs and Patent Appeals stated in *In re Langer*:

> As a matter of Patent Office practice, a specification which contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented <u>must</u> be taken as sufficient to satisfy the utility requirement of § 101 for the entire claimed subject matter <u>unless</u> there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope.

In re Langer, 183 USPQ 288, 297 (CCPA, 1974, emphasis in original). To overcome the presumption of sufficient utility as asserted by an applicant, the Examiner must carry the initial burden to make a *prima facie* showing of lack of utility and provide a sufficient evidentiary basis for the conclusion. In other words, the Examiner "must do more than merely question operability –[he] must set forth factual reasons which would lead one skilled in the art to question objective truth of the statement of operability." *In re Gaubert*, 187 USPQ 664, 666 (CCPA 1975).

In the present case, Applicants have asserted a specific and substantial utility in the specification and submitted Dr. Zoller's declaration and later-obtained experimental data which demonstrates that this asserted utility is credible to one of skill in the art and has been proven to be valid. In contrast, the Examiner has not provided any evidence or objective reason to overcome the

present patentable utility. Accordingly, Appellants respectfully submit that the rejection of claims 157-185 based on alleged lack of utility should be reversed.

Arguments Relating to 35 U.S.C. § 112 First Paragraph: Utility-Based Enablement Rejection of Claims 157-185

Claims 157-185 also stand rejected under 35 U.S.C. § 112 first paragraph for alleged inadequate enablement. This rejection is a utility-based enablement rejection. The Examiner stated that "since the claimed invention is not supported by a substantial asserted utility, for the reasons set forth above, one skilled in the art would not know how to use the claimed invention so that it would operate as intended without undue experimentation." (Advisory Action dated June 9, 2004, page 4, lines 20-23).

For the reasons set forth above, the Appealed claims satisfy the utility requirement under 35 U.S.C. § 101. The § 112 enablement rejection is based on the same reasoning as the § 101 rejection. Appellants therefore respectfully submit that for the same reasons set forth above in the traversal of the § 101 rejection, the utility-based enablement rejection is unsustainable and should therefore be reversed.

The Examiner further alleges that the Specification does not enable the genus of nucleic acid sequences encompassed by the claims on Appeal. The Advisory Action states that, "Applicant has not provided sufficient guidance as to how to make and use the encoded polypeptides which are not 100% identical to the polypeptide of SEQ ID NO: 8, but which still retain a desired property of the polypeptide of SEQ ID NO: 8" (Advisory Action page 5, lines 9-12) Appellants respectfully disagree.

Again Appellants respectfully submit that the genus of nucleic acid sequences embraced by the subject claims would be anticipated to encode polypeptides having a substantial degree of sequence identity with the endogenous hT2R61 polypeptide sequence (SEQ ID NO: 8). As noted in the

traversal of the § 101 rejection *supra*, the only independent claims which claim hT2R61 sequence variants (Claims 158-159) require:

(1) that the nucleic acid sequence specifically hybridize to SEQ ID NO: 7 under high stringency conditions (conditions recited in claim 158) and that it encode a functional bitter taste receptor (Claim 158); or

(2) that the nucleic acid encode a polypeptide that is at least 95% identical to the native hT2R61 polypeptide.

One skilled in the art, based on the teachings of the Specification and the claims would routinely be able to isolate nucleic acid sequences which hybridize to hT2R61 nucleic acid sequence (SEQ ID NO 7) or to synthesize polynucleotides which encode polypeptides that are at least 95% identical to hT2R61. In contrast to the final rejection, the genus of sequences encompassed by the claims is finite.

Moreover, the Specification provides substantial information relating to T2R assays that would enable one skilled in the art to screen these variant hT2R61 nucleic acid sequences and identity those variants which are functional, *i.e.,* bind the same bitter ligands which specifically interact with wild-type hT2R61 polypeptide.

Therefore, the Specification does teach a skilled artisan how to screen variant hT2R61 polypeptides according to the invention in order to assess whether such variants retain the desired function (specifically interact with hT2R61-specific bitter ligands).

It would be anticipated that these screening methods would not require undue experimentation because it would be anticipated that most of the genus of nucleic acid sequences embraced by the claims, would encode polypeptides that have a sequence having a high degree of sequence identity to hT2R61 (SEQ ID NO: 8) and therefore encode polypeptides having the same functional properties as native hT2R61. This would be anticipated since claim 158 (and claims dependent thereon require that the isolated sequence hybridize to the

endogenous hT2r61 nucleic acid sequence under high stringency hybridization conditions, and claims 159 and claims dependent thereon explicitly require that the variant polypeptide possess at least 95% sequence identity to hT2R61 (SEQ ID NO: 8). Based thereon, it would be anticipated that these nucleotide sequences would encode polypeptides having polypeptide sequences having a high degree of sequence identity with hT2R61.

Based on the foregoing, and for the same reasons set forth in Appellants' traversal of the § 101 rejection, the Specification would enable one skilled in the art to identity isolated nucleic acid sequences according to the invention which encode functional bitter taste receptors. Therefore reversal of the § 112 enablement rejection of claims 158-185 is respectfully believed to be in order.

Argument Relating to § 112 First Paragraph Written Description Rejection

The position of the Examiner is that the Specification does not reasonably establish that the inventors were in "possession" of the genus of nucleic acid sequences encompassed by the claims on Appeal. The Examiner has suggested that one skilled in the art "would not be able to make useful predictions as to the nucleotide positions or identities of those sequences based on the information in the specification."

Also, the Examiner states that the "instant disclosure of a single polypeptide, that of SEQ ID NO: 7, encoding a polypeptide with no instantly disclosed specific activities, does not adequately support the scope of the claimed genus." Appellants respectfully disagree.

Again, Appellants respectfully emphasize that the genus of nucleic acid sequences embraced by the subject claims would be anticipated to encode T2R variants that possess a substantial degree of sequence identity with the endogenous hT2R61 polypeptide (SEQ ID NO: 8).

Indeed, the only independent claims which encompass hT2R61 sequence variants (Claims 158-159) respectively require:

(1) that the nucleic acid sequence specifically hybridize to SEQ ID NO: 7 under high stringency conditions <u>and</u> encode a functional bitter taste receptor (Claim 158); or

(2) that the nucleic acid encode a polypeptide that is at least 95% identical to the native hT2R61 polypeptide that functions as a bitter taste receptor.

For the same reasons set forth above, one skilled in the art, based on the teachings of the Specification and claims would understand how to select nucleic acid sequences that hybridize to hT2R61 according to the recited stringent hybridization conditions (claim 158) or to synthesize polynucleotides which encode polypeptide that are at least 95% identical to hT2R61. (claim 158) In contrast to the final rejection, the genus of sequences encompassed by the claims is of a determinate scope.

Moreover, and in contrast to the final rejection, the Specification <u>does</u> provide both a structure and function for the claimed genus of nucleic acid sequences, namely they encode bitter taste receptor polypeptides (function) having a structure which is at least 95% identical to hT2R61 (independent claim 159), or a structure similar enough to permit the isolated nucleotide sequence to hybridize to SEQ ID NO: 7 under highly stringent hybridization conditions (claim 158).

Additionally, for the same reasons set forth above in the traversal of the § 101 rejection, the Specification further provides enough information to establish that Applicants were in possession of a functional human T2R (hT2R61) and further were in possession of assays that would be anticipated to identify bitter ligands that specifically interact with hT2R61 and variants thereof (SEQ ID NO: 8).

One skilled in the art, based on the teachings of the specification, would know how to screen variant hT2R61 polypeptides encoded by isolated nucleic acid sequences according to the claims and how to assess whether such variants retain the desired function (specifically interact with hT2R61-specific bitter ligands).

Moreover, the specification contains further information illustrating that the inventors were in possession of structural evidence demonstrating that they were in "possession" of the invention. Particularly, the application incorporates by reference two publications (Chandrashekar (Exhibit 1) and Adler (Exhibit 2)) which included saccharin in a compound library screened to identify potential T2R modulatory compounds (T2R61 was not included in the screened T2Rs.). Therefore, the Specification establishes that the inventors were in possession of information which would suggest that saccharin potentially should be screened against a T2R as a potential T2R modulatory compound. (This further would have been obvious, because saccharin was well known at the time this application was filed to elicit both sweet and bitter taste). Using such assays, saccharin and a very closely related derivative thereof have been shown to be hT2R61 ligands (Zoller Declaration ¶ 13). This publication incorporated by reference in its entirety in the as-filed specification further substantiates that the inventors were in possession of functional and structural information which would have resulted in the identification of hT2R61 variants which retain the desired function (bind hT2R61-specific bitter ligands)..

The written description rejection therefore should be reversed because Appellants have satisfied the controlling guidelines for a genus as set forth by the Federal Circuit in *University of California v. Eli Lilly & Co.*, 43 USPQ2d 1398 (Fed. Cir. 1997). As described by the Federal Circuit in *Lilly*, "[a] description of a genus of cDNAs may be achieved by means of ...a recitation of structural features common to members of the genus...." *Lilly*, 43 USPQ2d at 1406. Furthermore, the court in *Fiers v. Revel* stated that an adequate written

description "requires a precise definition, such as by structure, formula, chemical name, or physical properties." *Fiers*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993).

On the other hand, proper description of functional features of a claimed invention can play an important role in satisfying the written description requirement. The Federal Circuit recently state that "*Lilly*" did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular known structure." *Amgen Inc. v. Hoechst Marion Roussel, Inc.* 65 USPQ2d 1385, 1398 (Fed. Cir. 2003).

With regard to the claimed nucleic acids, the pending claims set forth both functional features, *e.g.*, encoding a bitter taste transduction GPCR that binds to and become activated by bitter ligands, and structural features, *e.g.*, encoding a GPCR comprising an amino acid sequence having a certain percentage identity to a reference amino acid sequence.

The percentage sequence identity of a polypeptide to a reference amino acid sequence is a physical/structural property of the nucleic acid encoding the polypeptide because the amino acid sequence of the polypeptide is determined by the polynucleotide sequence of the nucleic acid. Thus, the pending claims set forth commonly shared structural features of the claimed nucleic acids by describing the percentage amino acid sequence of the polypeptide encoded by the nucleic acids, or a nucleic acid sequence that possesses sufficient identity to SEQ ID NO: 7 so that it specifically hybridizes thereto according to the recited stringent hybridization conditions (Claim 158).

Commonly shared functional features of the claimed nucleic acids are also provided: each encodes a taste transduction GPCR that can bind to and become activated by bitter ligands. These functional features can be readily tested and verified by one of ordinary skill in the art using well established, routinely

practiced techniques as well as according to the teaching of the present specification (*See, e.g.,* pages 50-69 of the Specification). The reliability and reproducibility of these methods has moreover been demonstrated based on the fact that two different groups have used these assays and have identified bitter ligands, including saccharin, that specifically bind hT2R61 (See Exhibits 3 and 4).

Thus, both structural and functional features commonly shared by the claimed genus have been described in detail, which "clearly allow persons of ordinary skill in the art to recognize that [the applicant] invented what is claimed." *Vas-Cath Inc. v. Mahurkar*, 19 USPQ2d 1111, 1116 (Fed. Cir. 1991). Such description is consistent with the standards set forth in both *Lilly* and *Amgen*.

In summary, based on the foregoing arguments and analysis under *Lilly* and *Amgen* provided above, Appellants respectfully submit that the claimed invention within the current claim scope is properly described by the Specification as required by § 112 first paragraph. As such, reversal of the written description rejection of claims 158-159 and 164-185 is respectfully requested.

CONCLUSION

Based on the foregoing, Appellants respectfully submit that claims 158-185 comply with on the § 101 utility, § 112 enablement and § 112 written description guidelines because:

(i) it was reasonable for Applicants to conclude based on information contained in the as-filed application that the exemplified hT2R61 nucleic acid sequence encoded a bitter taste receptor;

(ii) it was reasonable for Applicants to conclude that polypeptides encoded by the claimed T2R61 nucleic acid sequences could predictably be used in high throughput screening assays to identify ligands that modulate bitter taste; absent undue experimentation, and

(iii) that the inventors' reasonable expectations have been confirmed by functional data obtained by them and another group demonstrating that the hT2R61 polypeptide, when screened against potential T2R ligands in high throughput screening assays, specifically binds to bitter ligands.

Therefore, reversal of the outstanding § 101 and § 112 rejections of claims 158-185 and the allowance of this application is respectfully believed to be in order. A Decision to that effect is respectfully requested.

This Appeal Brief is accompanied by a check in the amount of $170.00 in payment of the required appeal fee. This amount is believed to be correct, however, the Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, to Deposit Account No. 05-1323, Docket No.: 100337.54075US. A triplicate copy of this Appeal Brief is attached

Respectfully submitted,

November 15, 2004

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Claims Appendix

Pursuant to § 41-37(c)(1)(vii), a clean copy of all the claims on appeal in this application is set forth below:

158. An isolated nucleic acid molecule encoding a bitter taste receptor selected from the group consisting of

(i) an isolated nucleic acid sequence having the nucleic acid sequence contained in SEQ ID NO:7;

(ii) a nucleic acid sequence that encodes the bitter taste polypeptide contained in SEQ ID NO:8; and

(iii) an isolated DNA sequence that hybridizes under stringent hybridization conditions to the nucleic acid sequence contained in SEQ ID NO:7 wherein stringent hybridization conditions are hybridization in 5 x SSC, 1% SDS, incubated at 65°C and wash in 0.2 x 55C and 0.1% SDS at 65°C, wherein said hybridization and wash steps are each effected for at least 1 minute.

159. An isolated nucleic acid molecule encoding a bitter taste receptor polypeptide which polypeptide comprises at least 95% identity to the taste receptor polypeptide contained in SEQ ID NO:8, wherein sequence identity is determined by any one of the BLAST, BLAST 2.0 or PILE UP algorithms.

160. An isolated nucleic acid molecule which consists of the nucleic acid sequence contained in SEQ ID NO:7.

161. An isolated nucleic acid molecule which encodes the receptor polypeptide contained in SEQ ID NO:8 which is operably linked to a promoter that regulates the expression of said polypeptide.

162. The isolated nucleic acid molecule of claim 161 wherein said promoter is constitutive.

163. The isolated nucleic acid molecule of claim 161 wherein said promoter is regulatable.

164. An isolated nucleic acid sequence according to claim 159 which encodes a bitter taste receptor having at least 96% sequence identity to the polypeptide contained in SEQ ID NO:8.

165. An isolated nucleic acid sequence according to claim 159 which encodes a bitter taste receptor having at least 97% sequence identity to the polypeptide contained in SEQ ID NO:8.

166. An isolated nucleic acid sequence according to claim 159 which encodes a bitter taste receptor having at least 98% sequence identity to the polypeptide contained in SEQ ID NO:8.

167. An isolated nucleic acid sequence according to claim 159 which encodes a bitter taste receptor having at least 99% sequence identity to the polypeptide contained in SEQ ID NO:8.

168. An isolated nucleic acid molecule according to claim 159 which is operably linked to a promoter that regulates the transcription thereof.

169. The isolated nucleic acid molecule of claim 168 wherein said promoter is constitutive.

170. The isolated nucleic acid molecule of claim 168 wherein said promoter is regulatable.

171. An isolated nucleic acid sequence according to claim 158 attached to a nucleic acid sequence encoding a chaperone protein.

172. An expression vector containing an isolated nucleic acid molecule according to claim 158.

173. An expression vector containing an isolated nucleic acid molecule according to claim 159.

174. A cell which is transfected or transformed with an isolated nucleic acid molecule according to claim 158.

175. A cell which is transfected or transformed with an isolated nucleic acid molecule according to claim 159.

176. The cell of claim 174 which is selected from a mammalian, insect, amphibian, yeast, and bacterial cell.

177. The cell of claim 174 which is a mammalian cell.

178. The cell of claim 174 which additionally expresses a G protein.

179. The cell of claim 178 wherein said G protein is $G_{\alpha 15}$.

180. The cell of claim 177 which is an HEK-293 cell.

181. The cell of claim 175 which is selected from a mammalian, amphibian, yeast, insect and bacterial cell.

182. The cell of claim 181 which is a mammalian cell.

183. The cell of claim 175 which further expresses a G protein.

184. The cell of claim 183 wherein said G protein is $G_{\alpha 15}$.

185. The cell of claim 182 which is an HEK-293 cell.

Evidence Appendix

Pursuant to § 41.37(c)(1)(ix), this Appendix contains a listing of all evidence relied on in Appellants' Brief along with an identification of where this evidence was entered into the record. Copies of this evidence are also attached hereto.

1. Chandrashekar et al., *Cell* 100:703-11 (March 17, 2000). This reference was cited by the Examiner in the Office Action dated February 21, 2003. Additionally, this reference was incorporated by reference in its entirety at page 8, lines 13-15 of the Specification.

2. Adler et al., *Cell* Vol. 100:693-702 (March 17, 2000). This reference was cited in Zoller Declaration submitted with Applicants' § 116 Response dated May 4, 2004. This reference was considered by the Examiner (*See* page 3 of Advisory Action page 3, line 10-11, which states: "it is agreed that Chandrashekar and Adler provide the evidence that certain T2R members are, in fact, bitter taste receptors"). Additionally, this reference was incorporated by reference in its entirety at page 8, line 13-15 of the Specification.

3. "Activation of hT2R61 by Bitter Compounds",

This exhibit contains 4 pages of Experimental data confirming the functionality of hT2R61 receptor and its specific activation by bitter compounds including 6-nitrosaccharin). This evidence was provided with Applicants' resonse entitled "Response to Non-Final Office Action" dated August 21, 2003. (*See* attached postcard which refers to this evidence.) This evidence was considered by the Examiner (*See* page 11, lines 1-3 of Office Action dated November 14, 2003 which states, "Applicant's (sic) argues that Applicant's own subsequent research indicates that the polypeptide of SEQ ID NO: 8 is activated by two known bitter tastants, *e.g.* 6-3-gy-dinydro-isoguinolm-1 one (sic)."

4. "DECLARATION OF MARK ZOLLER, Ph.D"

This Declaration executed on May 13, 2004 was submitted with Applicants' response entitled "Response Pursuant to 37 C.F.R. § 1.116" dated May 14, 2004. This Declaration was entered and considered by the Examiner (*See* box 7 of Advisory Action dated June 9, 2004 and page 2, lines 15-16 of this Advisory Action which states the following):

> Applicant's arguments and the Zoller Declaration (May 14, 2004) evidence establishing SEQ ID NO: 8 as a functional bitter taste receptor have been fully considered.

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T2Rs Function as Bitter Taste Receptors

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Summary

Bitter taste perception provides animals with critical protection against ingestion of poisonous compounds. In the accompanying paper, we report the characterization of a large family of putative mammalian taste receptors (T2Rs). Here we use a heterologous expression system to show that specific T2Rs function as bitter taste receptors. A mouse T2R (mT2R-5) responds to the bitter tastant cycloheximide, and a human and a mouse receptor (hT2R-4 and mT2R-8) responded to denatonium and 6-n-propyl-2-thiouracil. Mice strains deficient in their ability to detect cycloheximide have amino acid substitutions in the *mT2R-5* gene; these changes render the receptor significantly less responsive to cycloheximide. We also expressed mT2R-5 in insect cells and demonstrate specific tastant-dependent activation of gustducin, a G protein implicated in bitter signaling. Since a single taste receptor cell expresses a large repertoire of T2Rs, these findings provide a plausible explanation for the uniform bitter taste that is evoked by many structurally unrelated toxic compounds.

Introduction

Mammals can perceive and distinguish between sweet, sour, bitter, and salty tastes (Kinnamon and Cummings, 1992; Lindemann, 1996a; Stewart et al., 1997). Of these four modalities, bitter perception has a particularly important role: many naturally poisonous substances taste bitter to humans, and virtually all animal species show an aversive response to such tastants (Garcia and Hankins, 1975; Glendinning, 1994; Glendinning et al., 1999), suggesting that bitter transduction evolved as a key defense mechanism against the ingestion of harmful substances.

The biology of bitter perception is very poorly understood; neither the sensory receptor cells nor the receptor molecules have been physiologically or molecularly defined (Lindemann, 1996b). However, several biochemical and physiological studies have suggested that bitter transduction in mammalian taste receptor cells is mediated by G proteins and G protein–coupled receptors (GPCRs) (Lindemann, 1996a; Wong et al., 1996). Because the universe of chemical compounds that evoke a bitter taste is structurally diverse, we reasoned that bitter receptors might encompass a large GPCR family with significant sequence variation. In the accompanying paper (Adler et al., 2000 [this issue of *Cell*]), we described the isolation of a novel family of 40–80 divergent GPCRs, T2Rs, selectively expressed in subsets of taste receptor cells of the tongue and palate epithelium. T2Rs in humans and mice are genetically linked to loci associated with bitter perception (Conneally et al., 1976; Capeless et al., 1992; Reed et al., 1999), and are selectively expressed in taste receptor cells that contain gustducin, a G protein α subunit implicated in bitter transduction (Wong et al., 1996; Ming et al., 1998). While the genetics, expression profile, and diversity of the T2R family support the proposal that T2Rs are taste receptors, rigorous demonstration of their role in taste transduction requires functional validation. Here we use a heterologous expression system to demonstrate that T2Rs function as receptors for bitter tastants. We analyzed mouse strains that differ in their recognition of various bitter compounds and show that mice that do not perceive low concentrations of cycloheximide contain missense mutations in the *mT2R-5* gene. These amino acid changes significantly reduce the sensitivity of the mT2R-5 receptor to cycloheximide. Notably, this sensitivity shift measured in cell-based assays closely mirrors the behavioral phenotype of the *Cyx*-deficient mice (Lush and Holland, 1988). The discovery of mammalian bitter receptors will help understand the biology of bitter perception, from transduction pathways in receptor cells to coding of bitter signals through the afferent sensory pathway.

Results and Discussion

Functional Expression of T2Rs

A difficulty in generating a cell-based reporter system to measure T2R activity is our poor understanding of the native signaling pathway. We therefore expressed T2Rs with Gα15, a G protein α subunit that has been shown to couple a wide range of receptors to phospholipase Cβ (Offermanns and Simon, 1995; Krautwurst et al., 1998). In this system, receptor activation leads to increases in intracellular calcium $[Ca^{2+}]_i$, which can be monitored at the single cell level using the FURA-2 calcium-indicator dye (Tsien et al., 1985). To test and optimize Gα15 coupling, we used two different GPCRs, a Gαi-coupled μ opioid receptor (Reisine, 1995) and a Gαq-coupled mGluR1 receptor (Masu et al., 1991). Transfection of these receptors into HEK-293 cells produced robust, agonist-selective, and Gα15-dependent Ca^{2+} responses (Figure 1). To assay T2R function, we initially generated four expression constructs containing epi-

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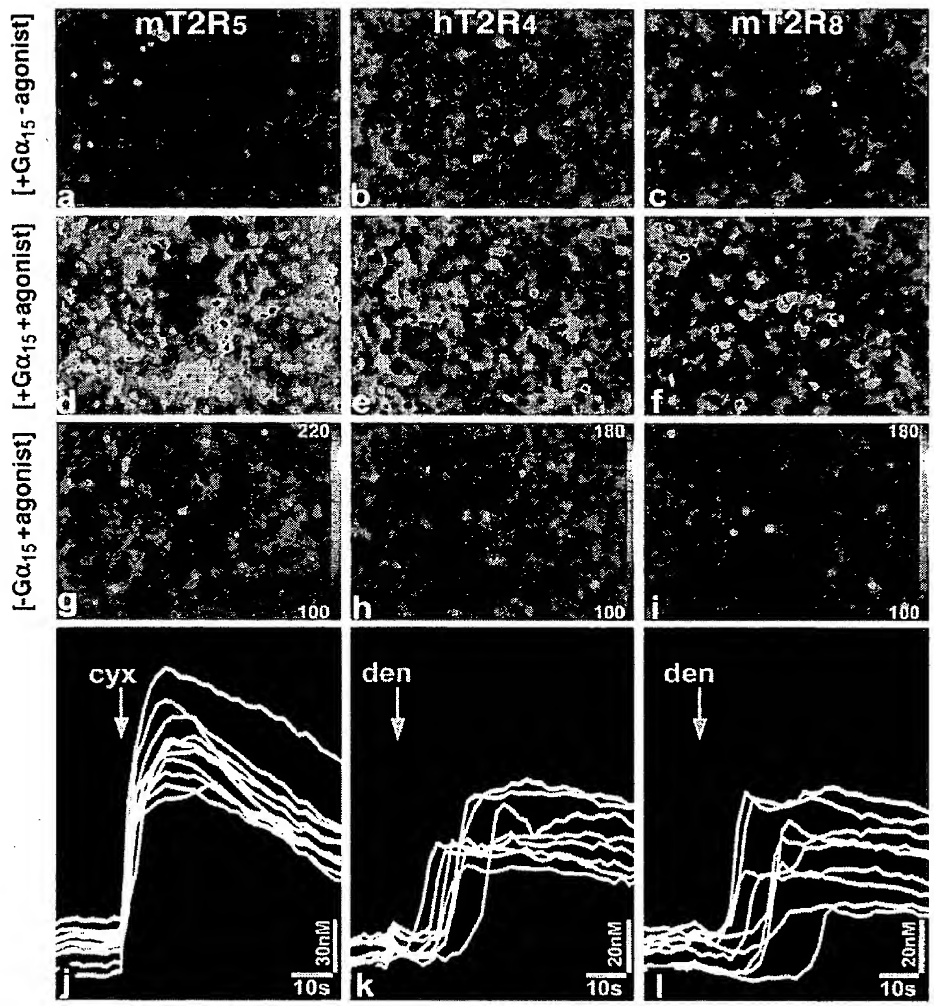

Figure 3. T2R Receptors Are Stimulated by Bitter Compounds

HEK-293 cells were transfected with rho-mT2R-5 (a, d, and g), rho-hT2R-4 (b, e, and h), and rho-mT2R-8 (c, f, and i). Cells expressing mT2R-5 were stimulated using 1.5 μM cycloheximide (d and g) and those expressing hT2R-4 and mT2R-8 with 1.5 mM denatonium (e, f, h, and i). No increase in $[Ca^{2+}]_i$ was observed in the absence of Gα15 (g–i); in contrast robust Gα15-dependent responses were observed in the presence of tastants (d–f); scales indicate $[Ca^{2+}]_i$ (nM) determined from FURA-2 emission ratios. Line traces (j–l) show the kinetics of the $[Ca^{2+}]_i$ changes for representative cells from panels (d–f); arrows indicate addition of tastants.

1α promoter and introduced as episomal plasmids into modified HEK-293 cells expressing Gα15 (PEAK^{rapid} cells; see Experimental Procedures).

We employed two parallel strategies to identify ligands for T2Rs. In one, we chose a random set of human, rat, and mouse T2R receptors, and individually tested them against a collection of 55 bitter and sweet tastants (see Experimental Procedures). We expected functional coupling to meet four criteria: tastant selectivity, temporal specificity, and receptor- and G protein-dependence. In the other, we used data on the genetics of bitter perception in mice to link candidate receptors with specific tastants.

Nearly 30 years ago, it was first reported that various inbred strains of mice differ in their sensitivity to the bitter compound sucrose-octaacetate (Warren and Lewis, 1970). Subsequently, a number of studies demonstrated that this strain difference was due to allelic variation at a single genetic locus (*Soa*) (Whitney and Harder, 1986; Capeless et al., 1992). These findings were extended to additional loci influencing sensitivity to various bitter tastants, including raffinose undecaacetate (*Rua*), cycloheximide (*Cyx*), copper glycinate (*Glb*), and quinine (*Qui*) (Lush, 1984, 1986; Lush and Holland, 1988). Genetic mapping experiments showed that the *Soa*, *Rua*, *Cyx*, *Qui*, and *Glb* loci are clustered at the distal end of chromosome 6 (Lush and Holland, 1988; Capeless et al., 1992). In the accompanying paper, we show that at least 25 mT2Rs colocalize with this mouse chromosome 6 bitter cluster (Adler et al., 2000). Therefore, we selected

4c and 4d). Notably, this dose response closely matches the sensitivity range of cycloheximide tasting in mice (Lush and Holland, 1988; see next section).

To examine the kinetics of the cycloheximide response, rho-mT2R-5 transfected cells were placed on a microperfusion chamber and superfused with test solutions under various conditions. Figure 4a shows robust transient responses to micromolar concentrations of cycloheximide that closely follow application of the stimulus (latency <1 s). As expected, when the tastant was removed, $[Ca^{2+}]_i$ returned to baseline. A prolonged exposure to cycloheximide (>10 s) resulted in adaptation: a fast increase of $[Ca^{2+}]_i$ followed by a gradual, but incomplete decline to the resting level (Figure 4a). Similarly, successive applications of cycloheximide led to significantly reduced responses, indicative of desensitization (Lefkowitz et al., 1992). This is likely to occur at the level of receptor, since responses of a cotransfected mGluR1 were not altered during the period of cycloheximide desensitization (data not shown).

Are other T2Rs also activated by bitter compounds? We assayed 11 rhodopsin-tagged human T2R receptors by individually transfecting them into HEK-293 cells expressing Gα15. Each transfected line was tested against a battery of bitter and sweet tastants, including amino acids, peptides, and other natural and synthetic compounds (see Experimental Procedures). We found that the intensely bitter tastant denatonium induced a significant transient increase in $[Ca^{2+}]_i$ in cells transfected with one of the human candidate taste receptors, hT2R-4, but not in control untransfected cells (Figure 3), or in cells transfected with other hT2Rs. The denatonium response had a strong dose dependency with a threshold of ~100 μM. While this response met the criteria of tastant selectivity, temporal specificity, and receptor- and Gα15-dependency, the threshold for activation was over two orders of magnitude higher than the human psychophysical threshold for denatonium (Saroli, 1984). This could be due to poor functioning of this receptor in the heterologous expression system, or perhaps humans express another higher affinity denatonium receptor. Interestingly, hT2R-4 displayed a limited range of promiscuity since it also responded to high concentrations of the bitter tastant 6-n-propyl-2-thiouracil (PROP; Figure 5).

If the responses of hT2R-4 reflect the in vivo function of this receptor, we hypothesized that similarly tuned receptors might be found in other species. The mouse receptor mT2R-8 is a likely ortholog of hT2R-4: they share ~70% identity, while the next closest receptor is only 40% identical; these two genes are contained in homologous genomic intervals (Adler et al., 2000). We generated a rho-mT2R-8 chimeric receptor and examined its response to a wide range of tastants. Indeed, mT2R-8, like its human counterpart, is activated by denatonium and by high concentrations of PROP (Figures 3 and 5). No other tastants elicited significant responses from cells expressing mT2R-8. Because these two receptors share only 70% identity, the similarity in their responses to bitter compounds attests to their role as orthologous bitter taste receptors.

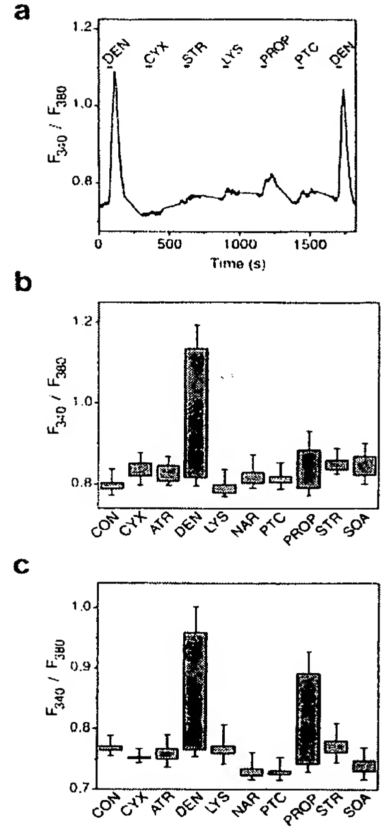

Figure 5. hT2R-4 and mT2R-8 Respond to Denatonium

HEK-293 cells expressing Gα15 were transiently transfected with hT2R-4 or mT2R-8 receptors and $[Ca^{2+}]_i$ was monitored as shown in Figure 3. (a) An increase in $[Ca^{2+}]_i$ could be induced by stimulation with denatonium but not by various other bitter compounds. Response profiles of (b) hT2R-4 and (c) mT2R-8 to a set of nine out of 55 different bitter and sweet tastants (see Experimental Procedures) are shown. CON refers to control buffer addition, NAR to 2 mM naringin and LYS to 5 mM lysine. Other abbreviations and concentrations are as reported in Figure 4. The mean FURA-2 fluorescence ratio (F_{340}/F_{380}) before and after ligand addition was obtained from 100 equal-sized areas that included all responding cells. The values represent the mean ± SE of at least six experiments.

Cycloheximide Nontaster Mice Have Mutations in the mT2R-5 Taste Receptor

Our demonstration that mT2R-5 functions as a high-affinity receptor for cycloheximide suggested that the *mT2R-5* gene might correspond to the *Cyx* locus. If this is true, we expected that either the expression profile or sequence of mT2R-5 might differ between strains categorized as *Cyx* tasters (DBA/2J) and nontasters (C57BL/6J) (Lush and Holland, 1988). In situ hybridizations to tissue sections demonstrated that the expression profile of mT2R-5 is indistinguishable between taster and nontaster strains (Figure 6). To determine the linkage between mT2R-5 and the *Cyx* locus, we identified polymorphisms in the *mT2R-5* gene and determined their distribution in a recombinant inbred panel

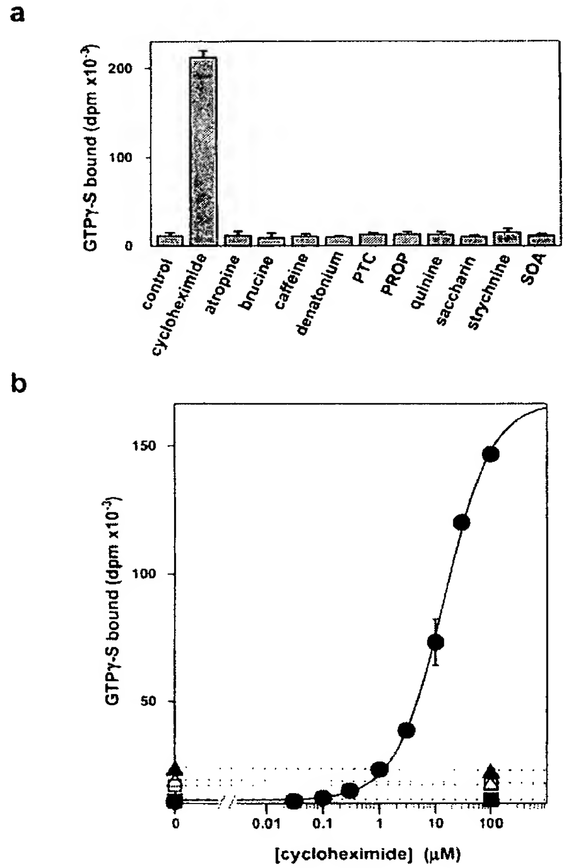

Figure 7. mT2R-5 Activates Gustducin in Response to Cycloheximide

(a) Insect larval cell membranes containing mT2R-5 activate gustducin in the presence 300 μM cycloheximide but not without ligand (control) or in the presence of 1 mM atropine, brucine, caffeine, denatonium, phenylthiocarbamide, 6-n-propyl thiouracil, quinine, saccharin, strychnine, sucrose octaacetate. (b) Cycloheximide concentration dependence of gustducin activation by mT2R-5 (filled circle) was fitted by single-site binding (K_d = 14.8 ± 0.9 μM). No cycloheximide-induced activity was detected in the presence of Gαo (filled triangle), Gαi (open triangle), Gαs (open square), or Gαq (filled square).

and gustducin were prepared using a baculovirus expression system. We incubated mT2R-5-containing membranes with various purified G proteins, including gustducin, and measured tastant-induced GTPγS binding (Hoon et al., 1995). Figure 7 shows the results of GTPγS binding assays, demonstrating exquisite cycloheximide-dependent coupling of mT2R-5 to gustducin. In contrast, no coupling was seen with Gαs, Gαi, Gαq, or Gαo. No significant GTPγS binding was observed in the absence of receptor, gustducin, or βγ heterodimers (data not shown). The high selectivity of T2R-5 for gustducin, and the exclusive expression of T2Rs in taste receptor cells that contain gustducin (Adler et al., 2000), affirm the hypothesis that T2Rs function as gustducin-linked taste receptors.

Concluding Remarks

To date, many putative taste receptors have been reported (Abe et al., 1993; Matsuoka et al., 1993; Ming et al., 1998; Hoon et al., 1999; Chaudhari et al., 2000). However, none have satisfied the requirements of rigorous biological verification: (1) demonstrated tissue and cell-specific expression, (2) functional validation, and (3) genetic corroboration. The T2R receptors presented in this and the accompanying paper were examined for all three criteria. First, we showed that T2Rs are selectively expressed in subsets of taste receptor cells of the tongue and palate epithelium. Second, three T2Rs (mT2R-5, hT2R-8, and mT2R-4) functioned as receptors for bitter tastants in heterologous cells. Third, polymorphisms in the mT2R-5 receptor were found to be associated with changes in bitter taste sensitivity to cycloheximide, both in vivo and in vitro. Thus, mT2R-5 is a strong candidate for *Cyx*. Furthermore, mT2R-5 selectively couples to gustducin, which has been implicated biochemically and genetically in taste transduction (Wong et al., 1996; Ming et al., 1998). Together, these results demonstrate that the T2R gene family contains functionally defined bitter taste receptors.

At present, we do not know what fraction of the available human and rodent receptors function in bitter transduction. However, our demonstration that all T2R-positive taste cells express multiple receptors suggests that T2R receptors may function in a similar taste modality. This is consistent with the observation that mammals can recognize a large number of bitter compounds, but do not discriminate between them (McBurney and Gent, 1979). Indeed, the two mouse receptors presented in this study (mT2R-5 and mT2R-8) respond to different bitter tastants and are expressed in combination with a number of other T2Rs in overlapping taste receptor cells (data not shown). Alternatively, if T2Rs respond to more than one modality, for example bitter and sweet then these cells would have to functionally segregate T2R receptors so as to maintain specificity and selectivity of signaling (Tsunoda et al., 1998).

A number of studies have shown that the oral cavity displays regional differences in sensitivity to the various taste modalities (Frank et al., 1983; Nejad, 1986; Frank, 1991). Our demonstration that T2Rs are expressed in all taste buds of circumvallate, foliate, and palate taste buds indicates that if there are significant differences in bitter sensitivity between these three regions, they may reflect events distal to tastant recognition.

The discovery of bitter taste receptors makes it possible to experimentally approach and elucidate critical aspects of the logic of bitter coding. For instance, it should be possible to genetically mark mT2R-expressing cells and examine their physiology and connectivity patterns. Similarly, it will be possible to knock out selective subsets of mT2R receptors and study the impact on bitter taste perception.

Taste receptor cells turn over throughout life (Beidler and Smallman, 1965). Therefore, synapses need to be continuously reestablished. It will be interesting to determine how this is achieved and whether nerve terminals provide any instructive signals for the expression of T2R receptors. The observation that taste buds degenerate when denervated and regenerate when the gustatory

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A Novel Family of Mammalian Taste Receptors

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Summary

In mammals, taste perception is a major mode of sensory input. We have identified a novel family of 40–80 human and rodent G protein–coupled receptors expressed in subsets of taste receptor cells of the tongue and palate epithelia. These candidate taste receptors (T2Rs) are organized in the genome in clusters and are genetically linked to loci that influence bitter perception in mice and humans. Notably, a single taste receptor cell expresses a large repertoire of T2Rs, suggesting that each cell may be capable of recognizing multiple tastants. T2Rs are exclusively expressed in taste receptor cells that contain the G protein α subunit gustducin, implying that they function as gustducin-linked receptors. In the accompanying paper, we demonstrate that T2Rs couple to gustducin in vitro, and respond to bitter tastants in a functional expression assay.

Introduction

Mammals taste many compounds but are believed to distinguish between only five basic taste modalities: sweet, bitter, sour, salty, and umami (the taste of monosodium glutamate). Although the discriminatory power of taste appears modest, it provides animals with valuable sensory information for the evaluation of food. The sense of taste evokes responses that range from innate behavioral actions such as aversion and attraction to food sources, to the pleasure of food consumption.

Mammalian taste receptor cells are clustered in taste buds, which are distributed on the surface of the tongue and palate. Each taste modality is thought to be mediated by distinct transduction pathways expressed in subsets of receptor cells (Kinnamon and Cummings, 1992; Lindemann, 1996; Stewart et al., 1997). Electrophysiological studies suggest that sour and salty tastants modulate taste receptor cell function by direct effects on specialized membrane channels (Heck et al., 1984; Brand et al., 1985; Avenet and Lindemann, 1988; Kinnamon et al., 1988; Gilbertson et al., 1992). In contrast, sweet, bitter, and umami taste transduction are believed to be mediated by G protein–coupled receptor (GPCR) signaling pathways (Striem et al., 1989; Wong et al., 1996; Chaudhari et al., 2000). These cell surface receptors interact with tastants and initiate signaling cascades that culminate in neurotransmitter release. Afferent nerve fibers from cranial nerve ganglia then relay the signals via the thalamus to cortical taste centers, where information is processed and integrated.

How does the brain interpret chemosensory information? Some of the most valuable insights into chemosensory coding have been derived from studies of olfactory reception in mice, worms, and flies. In mammals, individual olfactory neurons express only 1 of ~1000 different olfactory receptors, and all neurons expressing a common receptor project to the same set of glomeruli (reviewed by Mombaerts et al., 1996). Interestingly, a single olfactory receptor recognizes multiple odorants, and an odorant is recognized by multiple receptors (Malnic et al., 1999). Thus, mammals utilize combinatorial codes of glomeruli activation to respond to a wide diversity of odorants, and do so with exquisite discriminatory power (see for example Rubin and Katz, 1999). Worms also have hundreds of different receptors, but have only a few chemosensory neurons each expressing a large repertoire of receptor molecules (Troemel et al., 1995). Therefore, the system preserves the ability to respond to a wide diversity of odorants, but sacrifices discriminatory power. This simpler coding paradigm makes sense in an organism that needs to respond differentially to attractive and repulsive stimuli, but not between signals within each of these two modalities.

In contrast to the olfactory system, our understanding of taste coding and information processing is very limited, even at the basic cellular level. For example, it is not known whether individual taste receptor cells are tuned to specific or to many stimuli, or whether functionally similar cells are innervated by common fibers. Furthermore, while it is well established that taste buds from the different papillae in the tongue and palate epithelium exhibit specific taste sensitivities (Frank et al., 1983; Nejad, 1986; Frank, 1991), we do not understand how such differences are encoded in the organization and composition of the various taste buds (see for example Hoon et al., 1999).

We have been interested in basic questions of taste signal detection and information coding, and have focused primarily on sweet and bitter transduction. What are the receptors for sweet and bitter pathways? How is tastant specificity and taste discrimination accomplished? What is the topographic organization of sweet and bitter responding cells in the various taste buds and papillae? And, how is the information transmitted and encoded in the afferent nerves (i.e., are there specifically tuned lines)? Answering these questions would be aided by the isolation of genes involved in taste signaling, ideally taste receptors, that can be used to mark the cells, define the corresponding signaling pathways and

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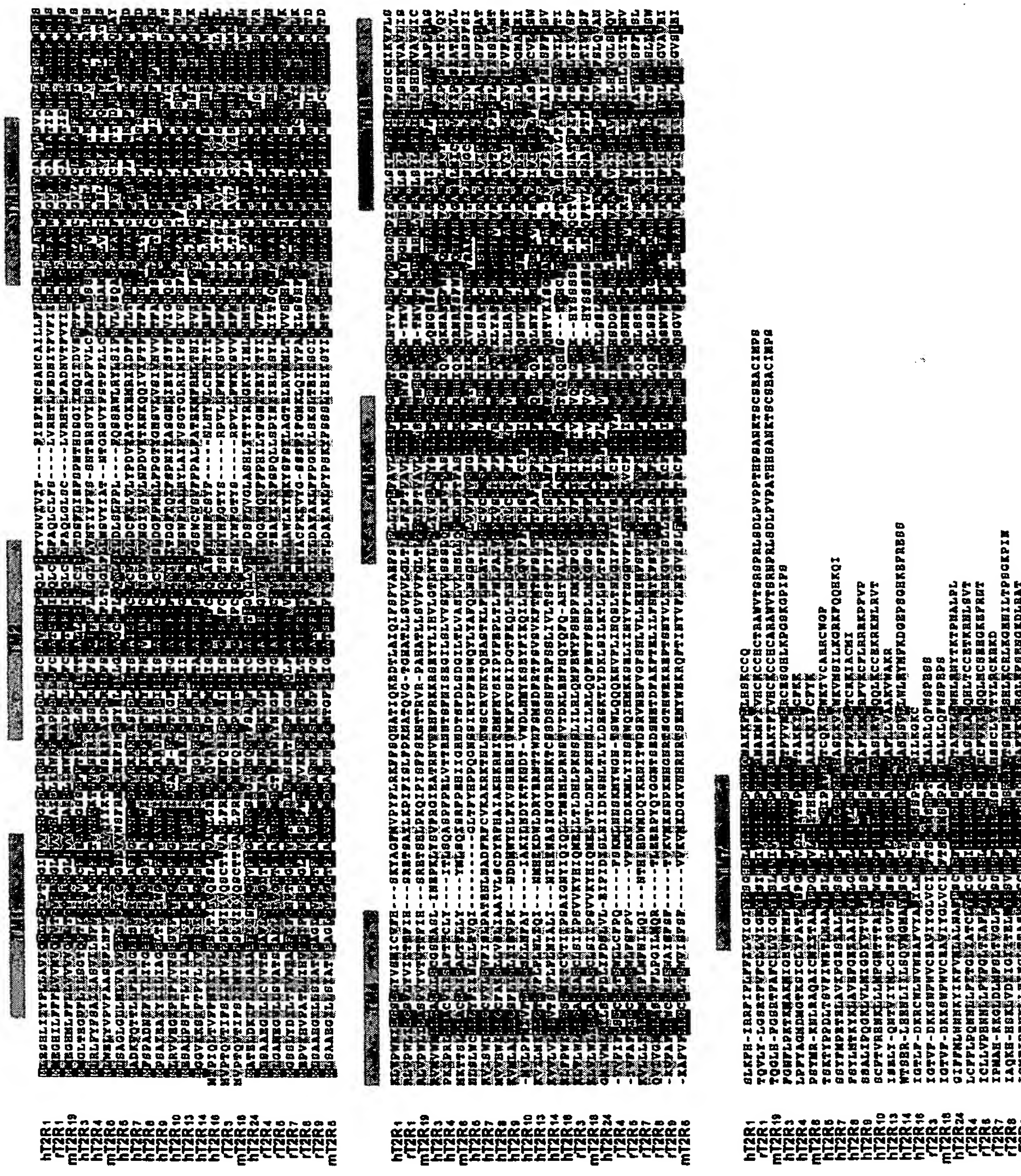

Figure 1. T2Rs Define a Novel GPCR Gene Family
Predicted amino acid sequences of representative human, rat, and mouse T2R genes (h, r, and mT2Rs) were aligned using ClustalW. Residues shaded in black are identical in at least half of the aligned sequences; conservative substitutions are highlighted in gray. Predicted transmembrane segments are indicated by bars above the sequence.

receptor specificity, generate topographic maps, and trace the respective neuronal connectivity circuits.

Recently, we isolated two novel GPCRs, initially called TR1 and TR2 (Hoon et al., 1999) and now referred to as T1R1 and T1R2, that are expressed in distinct subsets of taste receptor cells. While these may be receptors for sweet, bitter, or umami tastants, we reasoned that two receptors are too few to sample the chemically

diverse universe of sweet and bitter substances. In this and the accompanying paper (Chandrashekar et al., 2000 [this issue of *Cell*]), we report the isolation and characterization of a novel family of human and rodent taste receptors.

Results and Discussion

Identification of Novel Candidate Taste Receptors

To date, there are just a few G protein–coupled signaling molecules that have been implicated in taste transduction. T1R1 and T1R2 are putative taste receptors expressed in subsets of taste receptor cells of the tongue and palate epithelia (Hoon et al., 1999). In situ hybridization experiments showed that T1Rs are expressed in ~30% of the cells in the various taste buds. Gustducin is a G protein α subunit that is also found in a similar fraction of taste receptor cells of all taste buds (McLaughlin et al., 1992). Yet, for the most part, T1Rs are not coexpressed with gustducin, implying that there is an additional set of G protein–coupled receptors that must be expressed in gustducin-positive cells (Hoon et al., 1999). Genetic and biochemical evidence suggests that gustducin mediates some bitter responses (Wong et al., 1996; Ming et al., 1998). Thus, to try to identify receptors expressed in gustducin-positive cells, we searched for GPCRs in genomic intervals linked to bitter taste perception.

Recent genetic linkage studies in humans identified a locus at 5p15 that is associated with the ability to respond to the bitter substance 6-n-propyl-2-thiouracil (PROP; Reed et al., 1999). We reasoned that differences in PROP sensitivity may reflect functional differences in a bitter taste receptor, and searched DNA sequence databases for genes encoding candidate transmembrane proteins at this location. Analysis of open reading frames in 450 kb of DNA spanning six sequenced human genomic BAC clones from this interval identified a novel GPCR (T2R-1) at 5p15.2. T2R-1 has seven putative transmembrane segments as well as several conserved residues often present in GPCRs (Probst et al., 1992; Figure 1). Computer searches using T2R-1, and reiterated with T2R-1-related sequences, revealed 19 additional human receptors (12 full-length and 7 pseudogenes; see Figure 1). These novel receptors, referred to as T2Rs, define a novel family of GPCRs distantly related to V1R vomeronasal receptors (Dulac and Axel, 1995) and opsins (Figures 1 and 2). In contrast to T1Rs, which belong to the superfamily of GPCRs characterized by a large N-terminal domain (Hoon et al., 1999), the T2Rs have only a short extracellular N terminus. Individual members of the T2R family exhibit 30%–70% amino acid identity, and most share highly conserved sequence motifs in the first three and last transmembrane segments, and also in the second cytoplasmic loop (Figure 1, shaded boxes). The most divergent regions between T2Rs are the extracellular segments, extending partway into the transmembrane helices. We presume that the high degree of variability between T2Rs reflects the need to recognize many structurally diverse ligands. Like many other GPCR genes, T2Rs do not contain introns that interrupt coding regions.

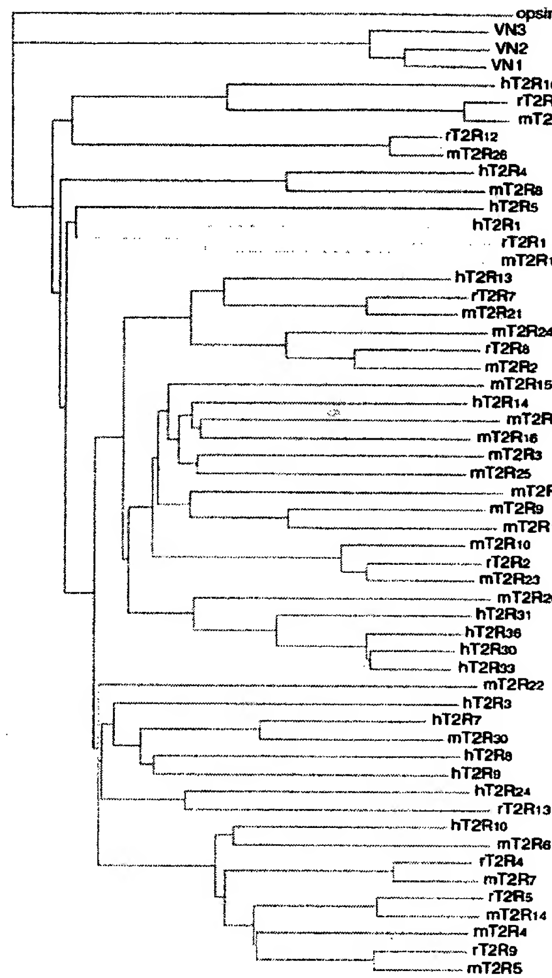

Figure 2. T2Rs Are a Structurally Diverse Family of Receptors Distantly Related to V1R Pheromone Receptors and Opsins

Sequence relationships between full-length human, mouse, and rat T2Rs, opsin, and V1R vomeronasal receptors (VN1–3) are displayed as a cladogram. The roots linking T2Rs are color-coded according to the chromosomal location of the various genes (see Figure 3). The identity between potentially orthologous rat and mouse T2Rs ranges from 74% for mT2R-2/rT2R-8 to 92% for mT2R-18/rT2R-3; identities between the three potentially orthologous pairs of human and mouse T2Rs are 67% for hT2R-4/mT2R-8, 51% for hT2R-16/mT2R-18, and 46% for hT2R-1/mT2R-19.

Organization of Human T2R Genes

The identified human T2R genes are localized on three chromosomes and are often organized as head-to-tail arrays (Figure 3). For example, four receptor genes are clustered within a single PAC clone from 7q31 and nine in a BAC clone from 12p13. There may be more T2Rs in these arrays, for example, several additional T2Rs are contained within partially sequenced BAC clones that overlap the 9 gene T2R cluster. Within an array, the similarity of receptors is highly variable, including both relatively related (e.g., T2R-13, T2R-14, and T2R-15), and highly divergent receptors (e.g., T2R-3 and T2R-4; Figures 2 and 3). This type of organization is mirrored

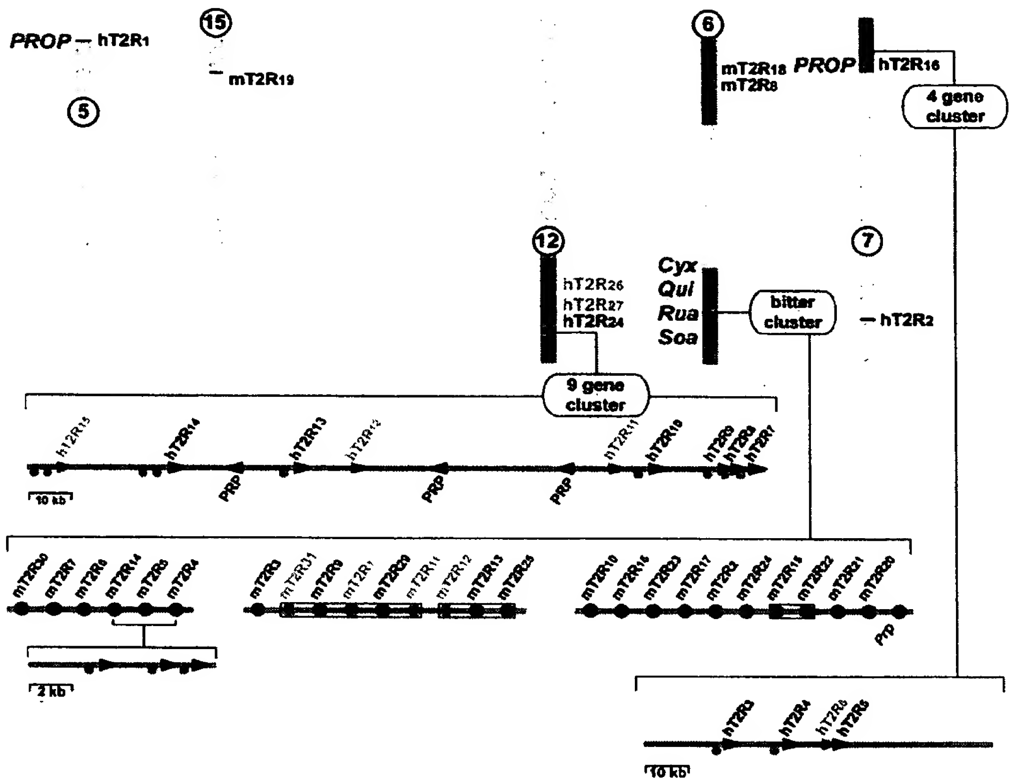

Figure 3. T2R Genes Map to Loci that Influence Bitter Taste

Schematic representation of human (chromosomes 5, 12, and 7) and mouse (15 and 6) chromosomes with homologous intervals color-coded; loci implicated in bitter perception are labeled red; T2R pseudogenes are gray. Also shown are expansions of the human 9 T2R gene cluster (accession number AC006518), human 4 T2R gene cluster (accession number AC004979), and three BAC contigs from the mouse chromosome 6 bitter cluster showing the order of some of the mT2R genes. Arrowheads indicate the direction of transcription. *PRP* refers to salivary proline-rich-protein genes (accession numbers M13058, K03202, and S79048). Offset colored dots represent a quasi-palindromic 18 bp sequence (e.g., ATTTGCATGGTTGCAAAT for hT2R-13) found in the 5′ upstream sequences of most T2R coding sequences. In general, this sequence is found 150–600 nt upstream of the putative initiator methionine. The mouse BAC contigs and mT2R genes within boxes are unordered and the relative orientation of the mT2R-4, -5, -14 cluster within the BAC contig is unknown.

in the mouse (see below) and resembles the genomic organization that has been observed for olfactory receptor genes in humans, mice, flies, and worms (Troemel et al., 1995; Sullivan et al., 1996; Rouquier et al., 1998; Clyne et al., 1999; Vosshall et al., 1999).

To get estimates of the size of this gene family, we examined various genomic resources. Analysis of the Genome Sequence Survey database (gss) yielded 12 partial T2R sequences. Because this database represents an essentially random sampling of ~14% of the human genome, there may be ~90 T2R genes in the human genome. Similar searches of the finished (nr) and unfinished high-throughput human genomic sequence databases (htgs) produced 36 full-length and 15 partial T2R sequences. These databases contain ~50% of the genome sequence, also pointing to ~100 T2R genes in the genome. Recognizing that this analysis may be inaccurate due to the quality of the available databases, and the clustered, nonrandom distribution of T2Rs in the human genome, we estimate that the T2R family consists of between 80 and 120 members. However, more than 1/3 of the full-length human T2Rs are pseudogenes; thus, the final number of functional human receptors may be significantly smaller (i.e., 40–80). This is similar to what has been observed for human olfactory receptors, where many of the genes appear to be pseudogenes (Rouquier et al., 1998).

T2R Genes Are Linked to Loci Involved in Bitter Taste

The genetics of sweet and bitter tasting has been extensively studied in mice, where a number of loci influencing responses to sweet and bitter tastants have been mapped by behavioral taste-choice assays (Warren and Lewis, 1970; Fuller, 1974). The distal end of mouse chromosome 6 contains a cluster of bitter genes that includes *Soa* (for sucrose octaacetate; Capeless et al., 1992), *Rua* (raffinose undecaacetate; Lush, 1986), *Cyx* (cycloheximide; Lush and Holland, 1988), and *Qui* (quinine; Lush, 1984). Recombination studies indicated that these four loci are closely linked to each other, and to *Prp* (salivary proline rich protein; Figure 3; Azen et al., 1986). Notably, the human 9 gene T2R cluster contains three interspersed *PRP* genes, and maps to an interval that is homologous with the mouse chromosome 6 bitter

cluster, thus raising the possibility that T2Rs are bitter taste receptors.

To define the relationship between the mouse chromosome 6 bitter cluster and T2Rs, we isolated a large number of mouse T2R genes and examined their genomic organization and physical and genetic map locations. We used human T2Rs to screen mouse genomic libraries and isolated 61 BAC clones containing 28 mouse T2Rs. The mouse and human receptors display significant amino acid sequence divergence (see Figures 1 and 2), but share the sequence motifs common to members of this novel family of receptors. Radiation hybrid and recombinant inbred strain mapping studies showed that these mouse genes are clustered at only a few genomic locations (Figure 3). Remarkably, each genomic interval containing mouse T2Rs is homologous to one containing its closest human counterpart: mT2R-8 and hT2R-4, mT2R-18 and hT2R-16, and mT2R-19 and hT2R-1. Of these three sets of potentially orthologous pairs of human/mouse receptors, both the human *T2R-1* and *T2R-16* genes map to locations implicated in human bitter perception (Conneally et al., 1976; Reed et al., 1999). The remaining 25 mT2Rs all map to the distal end of chromosome 6 and are represented by three BAC contigs spanning at least 400 kb.

Since *Prp* and the bitter-cluster also map to the distal end of mouse chromosome 6, we examined whether they localize within this array of T2Rs. Analysis of a DBA/2 × C57BL/6 recombinant inbred panel revealed that receptors within all three BAC contigs cosegregate with *Prp* and the bitter cluster (data not shown). We also isolated the mouse *Prp* gene (accession number M23236, containing *D6Mit13*) and showed that it lies within the large chromosome 6 T2R cluster (Figure 3). These results demonstrate that T2Rs are intimately linked to loci implicated in bitter perception, and substantiate the postulate that T2Rs may function as taste receptors.

T2Rs Are Expressed in Taste Receptor Cells

If T2Rs function as taste receptors, they should be expressed in taste receptor cells. The lingual epithelium contains taste buds in three types of papillae: circumvallate papillae at the very back of the tongue, foliate papillae at the posterior lateral edge of the tongue, and fungiform papillae dispersed throughout the front half of the tongue surface. Other parts of the oral cavity also have taste buds; these are particularly prominent in the palate epithelium in an area known as the geschmackstreifen and in the epiglottis (Figure 4). To examine the patterns of expression of T2Rs, we performed in situ hybridizations to sections of various taste papillae. To ensure that we tested probes expressed in taste tissue, we screened a rat circumvallate cDNA library. We isolated 14 rat T2Rs cDNAs, each of which is an ortholog of a mouse genomic clone (Figure 2).

T2Rs are selectively expressed in subsets of taste receptor cells of the tongue and palate epithelium. Figures 5a–5e show representative sections of rat circumvallate papilla taste buds hybridized with antisense cRNA probes to five different T2Rs. Each receptor hybridizes to an average of two cells per taste bud per section. Since our sections contain 1/5–1/3 the depth

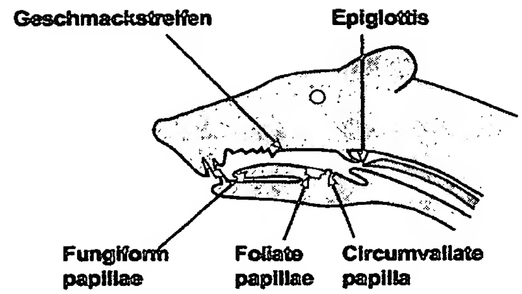

Figure 4. Functional Anatomy of the Rodent Oral Cavity
The diagram shows a drawing of a rodent head highlighting regions containing taste buds. We thank E. A. for modeling for this diagram.

of a taste bud, this reflects a total of 6–10 positive cells/taste bud/probe (or about 15% of the cells in a taste bud). Examination of serial sections demonstrated that all of the taste buds of the circumvallate papilla contain cells that are positive for each of these probes. Thus far, we have observed comparable results with 11 rat T2Rs, and in mouse sections hybridized with 17 different mT2R probes (data not shown).

Similar studies in foliate, geschmackstreifen, and epiglottis taste buds demonstrated that each receptor probe also labels ~15% of the cells in every taste bud (Figures 5f–5h). In contrast, T2Rs are rarely expressed in fungiform papillae. We examined hundreds of fungiform taste buds using 11 different T2R probes and found that less than 10% of all fungiform papillae contain T2R-expressing cells. Interestingly, the few fungiform taste buds that do express T2Rs regularly contain multiple positive cells (see Figure 5i). In fact, the number of positive cells in these papillae is not significantly different from that seen in taste buds from other regions of the oral cavity. Furthermore, fungiform papillae that contain T2R-expressing cells generally appear clustered. This unexpected finding may provide an important clue about the logic of taste coding. It is known that single fibers of the chorda tympani nerve innervate multiple cells in a fungiform taste bud, and that the same fiber often projects to neighboring papillae (Miller, 1974). Perhaps the nonrandom distribution of T2R-positive taste receptor cells and taste buds in fungiform papillae reflect a map of connectivity between similar cells.

Individual Receptor Cells Express Multiple T2R Receptors

We have shown that any one T2R is expressed in ~15% of the cells of circumvallate, foliate, and palate taste buds. Given that there are over 30 T2Rs in the rodent genome, a taste cell must express more than one receptor. But how many receptors are expressed in any cell and what fraction of taste receptor cells express T2Rs? We compared the number of circumvallate cells labeled with various mixes of 2, 5, or 10 receptors with those labeled with the corresponding individual probes (Figure 6). By counting positive cells in multiple serial sections, we determined that the number of taste cells labeled with the mixed probes (~20%) was only slightly larger than that labeled by any individual receptor (~15%;

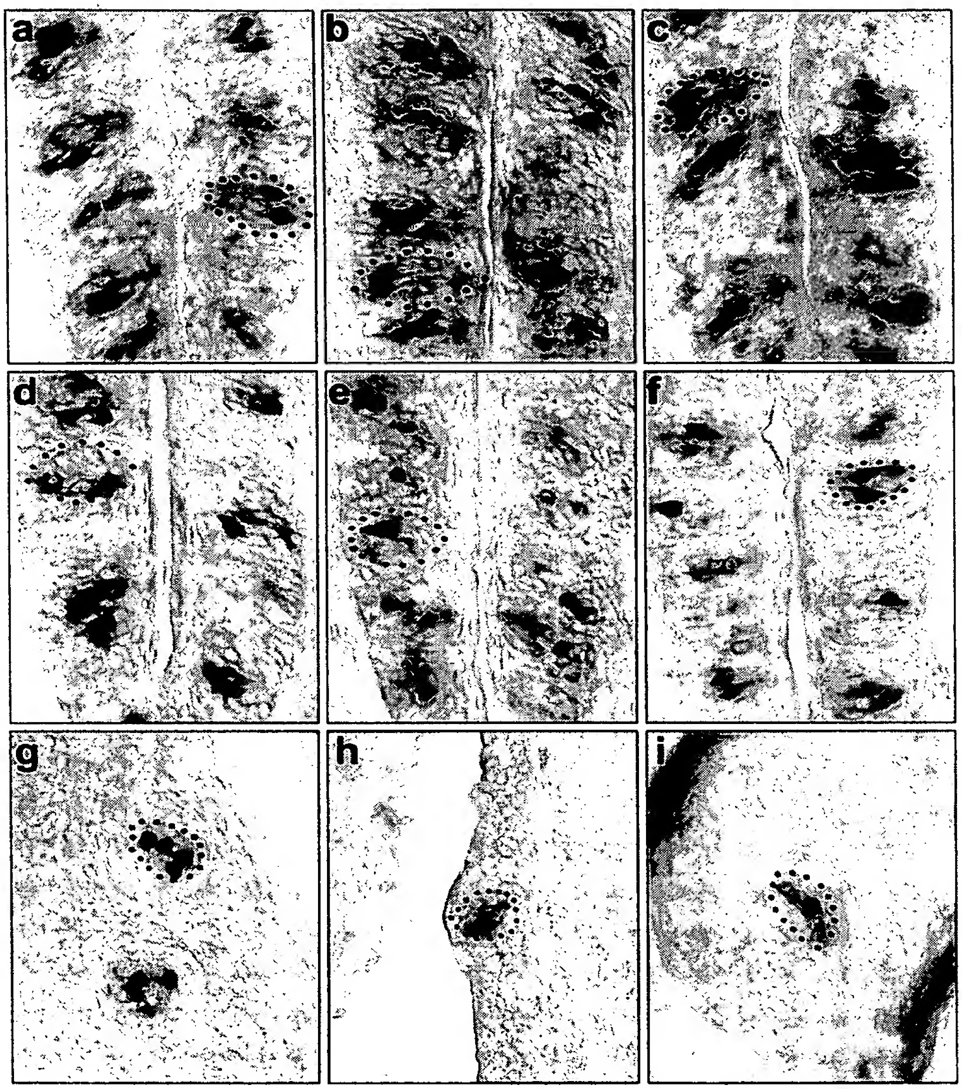

Figure 5. Expression of T2Rs in Subsets of Taste Receptor Cells

In situ hybridizations with T2R digoxigenin-labeled antisense RNA probes demonstrated that members of this family of receptors are expressed in subsets of taste receptor cells. All rat circumvallate taste buds contain cells expressing T2Rs: (a) rT2R-7, (b) rT2R-8, (c) rT2R-3, (d) rT2R-2, (e) rT2R-4. T2Rs are also expressed in all taste buds in the foliate papillae, geschmackstreifen and epiglottis: (f) foliate with rT2R-7, (g) geschmackstreifen with rT2R-3, and (h) epiglottis with rT2R-7. In contrast, less than 10% of all fungiform papillae contain T2R-expressing cells. However, the few fungiform taste buds that express T2Rs regularly contain multiple positive cells (i) (distribution of T2R-positive cells in sections of 400 fungiform taste buds: 3.5% one labeled cell, 5.25% two labeled cells, and 0.75% three or more labeled cells per section). The dotted lines indicate the outline of a sample taste bud.

compare Figures 5 and 6). Not surprisingly, the signal intensity was significantly enhanced in the mixed probe hybridizations. Similar results were observed in taste buds from other regions of the oral cavity including the fungiform papillae. To directly demonstrate coexpression we performed two-color double-label in situ hybridization experiments using a collection of differentially labeled cRNA probes. As expected, the majority of cells expressed multiple receptors (Figure 6d).

Our data on the expression patterns of T2Rs provide important insight into the organization of the taste system. First, as initially demonstrated with the T1Rs, there are marked topographic differences in the expression patterns of candidate signaling molecules in the various taste buds and papillae. Second, the complexity of the receptor repertoire is significantly larger than previously thought. Third, each cell expresses multiple receptors. Moreover, the demonstration that different mixtures of 2 or 5 probes detected as many positive cells as the mix of 10 suggests that each positive cell expresses nearly the full complement of T2Rs. If we assume that each receptor signals via the same pathway, and that the patterns of receptor expression delineate the logic of taste coding, these results indicate that there would

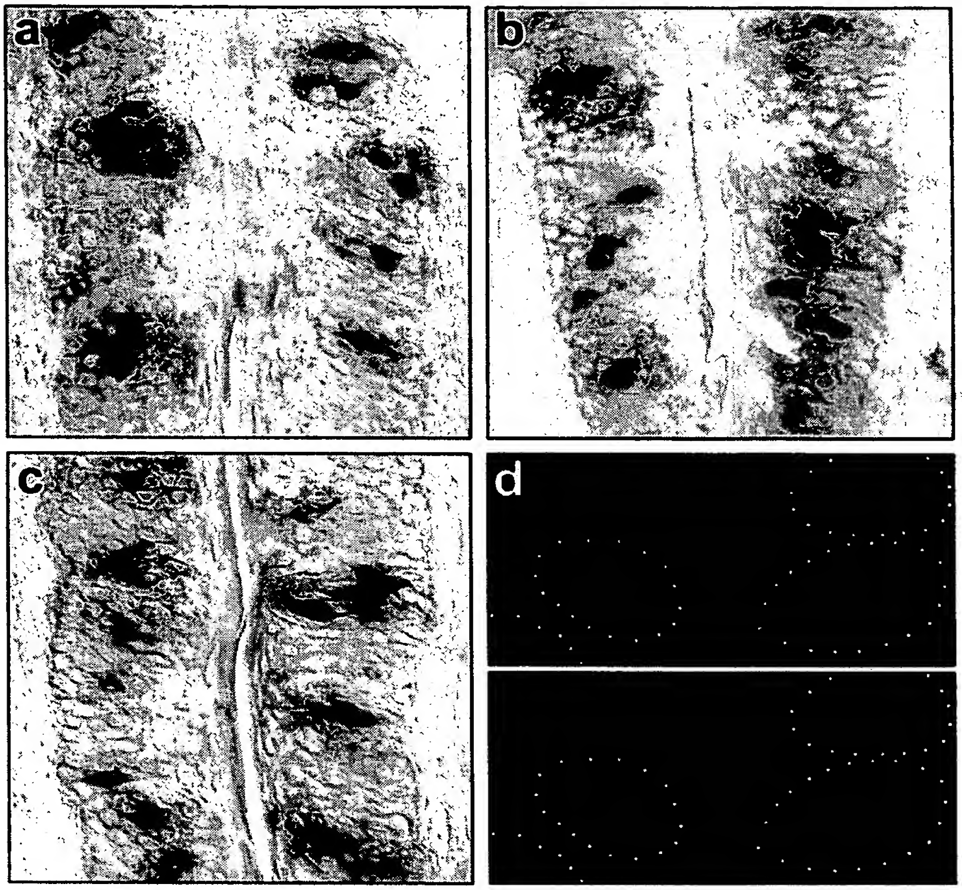

Figure 6. Many T2Rs Are Coexpressed in the Same Taste Receptor Cell

Mixtures of (a) 2, (b) 5, or (c) 10 T2R probes hybridized to only very few more cells than detected by any individual probe in circumvallate taste buds (see Figure 5 and Experimental Procedures for list of probes). Similar results were obtained in taste buds from other regions of the oral cavity. Double-label fluorescent in situ hybridizations (d) directly demonstrated coexpression of T2R-3 (green) and T2R-7 (red) in the same taste receptor cells; other mixtures of receptors produced equivalent results. The dotted lines outline the approximate area of sectioned taste buds.

be limited functional discrimination between T2R-positive cells.

T2R Genes Are Selectively Expressed in Gustducin-Expressing Cells

Previously, we have shown that T1Rs are expressed in ~30% of taste receptor cells. In situ hybridizations with differentially labeled T1R and T2R probes showed that there is no overlap in the expression of these two classes of receptors (Figure 7d). Gustducin is also expressed in a large subset of taste receptor cells, but for the most part is not coexpressed with T1Rs (Hoon et al., 1999). To determine if T2Rs are expressed in gustducin cells, we performed in situ hybridizations using differentially labeled T2Rs and gustducin riboprobes. Figure 7 demonstrates that T2Rs are exclusively expressed in gustducin-positive cells of the tongue and palate taste buds.

Do all gustducin-positive cells express T2Rs? Approximately 1/3 of the gustducin cells in the circumvallate, foliate, and palate taste buds did not label with a mix of 10 T2R probes (Figure 7). These cells may express other, perhaps more distantly related receptors, or could be at a different developmental stage. In fungiform taste buds the situation is quite different. Since only 10% of fungiform taste buds contain T2R-positive cells, the great majority of gustducin-positive cells in the front of the tongue do not coexpress members of the T2R family of receptors. While it is formally possible that fungiform receptor cells express T2Rs at levels below our limits of detection, we do not believe this to be the case. First, even when we used mixed probes and extended developing times, we did not detect additional positive cells. Second, PCR amplification reactions using T2R-specific primers on fungiform taste buds did not reveal a population of rarely expressed T2Rs. Third, the few fungiform taste buds that express T2Rs are positive for the full repertoire of probes, suggesting that all receptors are also expressed in the front of the tongue, but in a much smaller subset of taste buds. Therefore, there is likely to be an additional set of receptors expressed

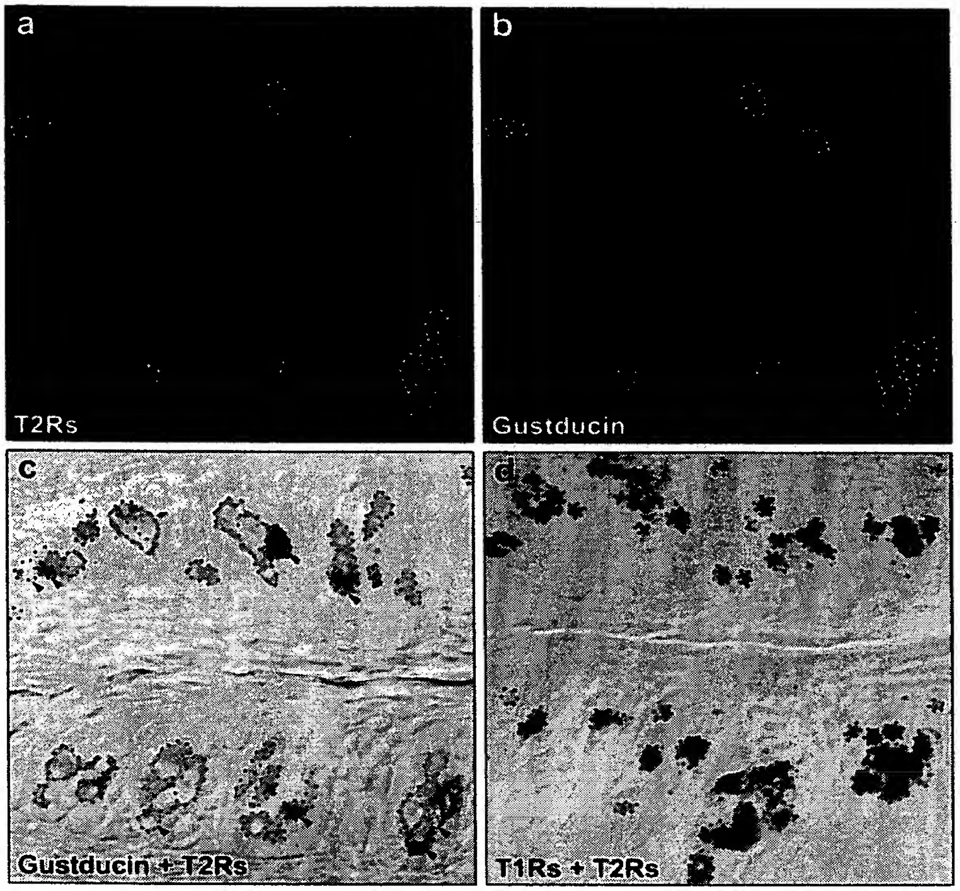

Figure 7. T2Rs Are Expressed in Taste Receptor Cells that Contain Gustducin

Double-label fluorescent in situ hybridizations were used to examine the expression of T2Rs with gustducin and T1Rs. (a) T2Rs are expressed in the same cells as (b) gustducin, as shown in (c) where the two-channel fluorescent image (1 μm optical section of a rat cicumvallate papilla) is overlaid on a difference interference contrast image. The dotted lines outline the approximate area of labeled taste receptor cells; arrows indicate gustducin-expressing cells that do not contain T2Rs. In contrast, (d) shows that T1Rs (red) are expressed in different subset of taste receptor cells from T2Rs (green).

in the gustducin-positive cells of fungiform papillae (see concluding remarks). Interestingly, gustducin is expressed outside taste receptor cells in isolated cells in the gastrointestinal tract (Hofer et al., 1996; Hofer and Drenckhahn, 1998), trachea, pharynx, nasal respiratory epithelium, ducts of salivary glands, and vomeronasal organ (data not shown). Some of these cells also express a small subset T2Rs, further supporting the idea that T2Rs are gustducin-linked receptors (data not shown). It will be interesting to determine whether these cells play a role in chemoreception.

It has been proposed that gustducin is involved in bitter and sweet transduction since gustducin knockout mice show decreased sensitivity to some sweet and bitter tastants (Wong et al., 1996). In addition, gustducin can be activated in vitro by stimulating taste membranes with bitter compounds, likely through the activation of bitter receptors (Ming et al., 1998). While our studies do not directly address the function of gustducin, the demonstration that T2Rs are expressed selectively in gustducin-positive cells is consistent with the proposal that they function as gustducin-linked taste receptors (see Chandrashekar et al., 2000).

Concluding Remarks

In this paper, we describe the identification of a novel family of GPCRs, T2Rs, selectively expressed in taste receptor cells of the tongue and palate epithelium. T2R receptors map to loci that have been reported to influence bitter taste perception in humans and mice, suggesting they function as bitter receptors. Numerous genetic and psychophysical studies point to different receptors for various types of bitter compounds (McBurney et al., 1972; Lush and Holland, 1988). However, perception of bitter compounds appears uniform to a human subject. Our finding that each taste receptor cell expresses a large number of T2Rs is consistent with the observation that mammals are capable of recognizing a wide range of bitter substances, but not distinguishing between them. In contrast, the distinct expression patterns of T1Rs and T2Rs suggest that these receptor families may encode different modalities.

How might coexpression of T2Rs be controlled? One possibility is that the clustering of genes allows coordinate regulation by the use of shared regulatory sequences. Another is that these genes have separate, but similar controlling elements. Notably, we found a common sequence motif (see Figure 3) present upstream of the initiator methionine in the majority of the human T2R genes. It should be possible to genetically manipulate this sequence and define its impact on the expression of T2Rs. This could be best accomplished in the mT2R-4, mT2R-5, and mT2R-14 cluster, where all three genes are organized as a head-to-tail array within a single 6 kb DNA fragment (Figure 3).

T2Rs are expressed exclusively in gustducin-positive cells, suggesting that these are gustducin-linked receptors. However, not all gustducin-positive cells express T2Rs, consistent with the proposal of multiple functions for gustducin (Wong et al., 1996). This is best illustrated in fungiform papillae, where only a minor fraction of gustducin-positive cells express T2Rs. In the accompanying paper (Chandrashekar et al., 2000), we directly demonstrate that T2Rs couple to gustducin and function as bitter receptors. The identification of candidate bitter taste receptors opens novel avenues into our understanding of taste biology, and provides a rational strategy for isolating bitter antagonists and modulating mammalian taste perception.

Experimental Procedures

Molecular Cloning of Taste Receptors

Human T2R-1 was discovered as an open reading frame with limited homology to GPCRs in a BAC (accession number AC003015) from 5p15.2. Additional T2Rs (numbered in order of discovery) were identified by reiterated sequence searches of DNA sequence databases. Full-length hT2Rs were isolated by PCR amplification of genomic DNA, and used to probe a rat circumvallate cDNA library (Hoon et al., 1999) and mouse BAC filter arrays (Genome Systems) at low stringency (50°C–55°C wash in 1× SSC). Southern hybridization experiments were used to identify a nonredundant set of positive BACs and to order overlapping BACs. Mouse T2Rs were mapped using a mouse/hamster radiation hybrid panel (Research Genetics) and by examining the strain distribution pattern of single nucleotide polymorphisms in a panel of C57BL/6J × DBA/2J recombinant inbred lines (Jackson Laboratory).

In Situ Hybridization

Tissue was obtained from adult rats and mice. No sex-specific differences of expression patterns were observed, therefore male and female animals were used interchangeably. For foliate sections, no differences in expression pattern were observed between the papillae. Fresh frozen sections (16 μm/section) were attached to silanized slides and prepared for in situ hybridization as described previously (Hoon et al., 1999). All in situ hybridizations were carried out at high stringency (hybridization, 5× SSC, 50% formamide, 65°C–72°C; washing, 0.2× SSC, 72°C). For single-label detection, signals were developed using alkaline-phosphatase conjugated antibodies to digoxigenin and standard chromogenic substrates (Boehringer Mannheim). Where possible, probes contained extensive 3′-nontranslated sequence to minimize potential cross-hybridization between T2Rs. The probes did not cross-hybridize at the stringency used for in situ hybridization. Control hybridizations with sense probes produced no specific signals in any of the taste papillae, while hybridization with a cDNA encoding a Gαi subunit demonstrated uniform labeling in all taste cells of all taste buds (data not shown). In all cases we examined at least 50 taste buds derived from a minimum of 3 animals. Quantitative studies were based on examination of 16 μm serial sections through various papillae. Cells were counted based on the position of their nucleus as previously described (Boughter et al., 1997). Experiments shown in Figure 6 used the following probes: (a) T2R-3 and T2R-7; (b) T2R-4, T2R-5, T2R-6, T2R-8, and T2R-12; (c) T2R-1, T2R-2, T2R-3, T2R-4, T2R-5, T2R-6, T2R-7, T2R-8, T2R-10, and T2R-12. Identical results were obtained with four additional combinations of two and nine additional combinations of five receptors (data not shown). Northern analysis and in situ hybridization demonstrated that T2Rs are not widely expressed outside taste tissue (data not shown). For double-label fluorescent detection, probes were labeled either with fluorescein or with digoxigenin. At least 50 taste buds from three different animals were analyzed. An alkaline-phosphatase conjugated anti-fluorescein antibody (Amersham) and a horseradish-peroxidase conjugated anti-digoxigenin antibody were used in combination with fast-red and tyramide fluorogenic substrates (Boehringer Mannheim and New England Nuclear). Confocal images were obtained with a Leica TSC confocal microscope using an argon-krypton laser; 1 μm optical sections were recorded to ensure that any overlapping signal originated from single cells.

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GenBank Accession Numbers

GenBank accession numbers for T2Rs are AF227129–AF227149 and AF240765–AF240768.

Activation of hT2R61 by bitter compounds
(for WO03006482A2 (T2R Blocker) patent application)
Alexey Pronin
Walter Keung

Example I: hT2R61 Responds to Bitter Compounds

Figure A contains the results of a GTPγS binding assay that shows that hT2R61 is activated by 6-nitrosaccharin but not 4-nitrosaccharin. At tested concentrations (0.5-2 mM) 6-nitrosaccharin is bitter to humans whereas 4-nitrosaccharin is not (Hamor, 1961). Figure A also demonstrates that a different hT2R, hT2R64 is not activated by 6-nitrosaccharin under the same conditions.

Figure A

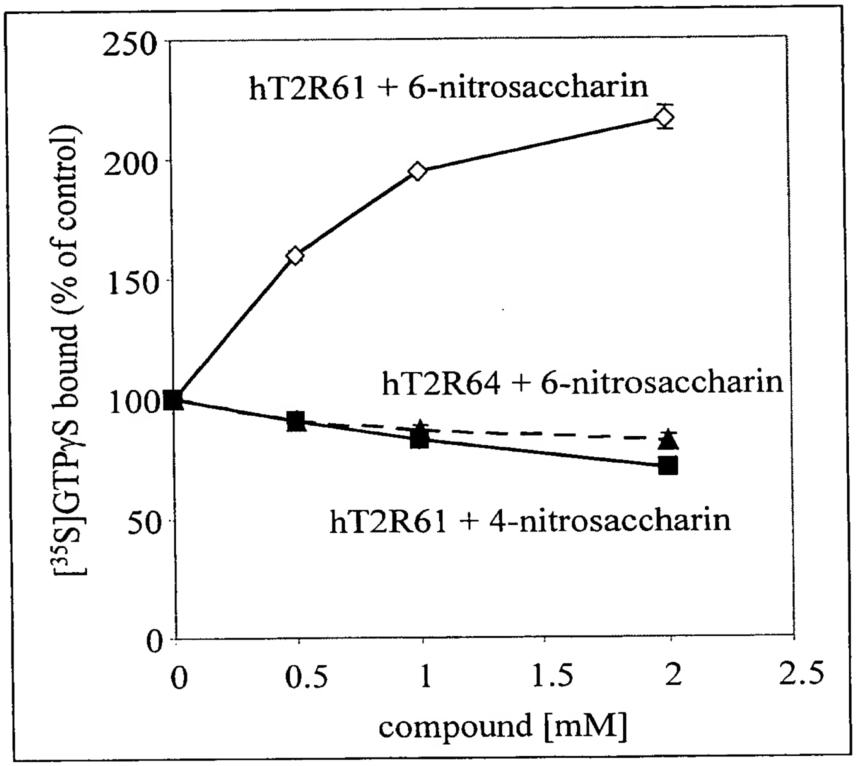

Figure A. 6-nitrosaccharin is activated by hT2R61 but not 4-nitrosaccharin. Activity of hT2R61 and hT2R64 was determined using GTPγS binding assay either in the absence or presence of indicated concentrations of 6-nitrosaccharin or 4-nitrosaccharin. The activity is expressed as a percentage of activity in the absence of added test compounds.

Example II: Identification of novel hT2R61 agonists of the 3,4-dihydro-isoquinolin-1-one class

Screening of over 15,000 compounds using the GTPγS binding assay identified novel molecules that specifically activate hT2R61. Figure B contains the results of a GTPγS

binding assay that shows that hT2R61 is activated by compounds from 3,4-dihydro-isoquinolin-1-one class:
#2623627 - 4-(4-Benzo[1,3]dioxol-5-ylmethyl-piperazine-1-carbonyl)-3-(4-methoxy-phenyl)-2-methyl-3,4-dihydro-2H-isoquinolin-1-one
#2623617 - 3-(4-Methoxy-phenyl)-2-methyl-4-(4-phenyl-piperazine-1-carbonyl)-3,4-dihydro-2H-isoquinolin-1-one

Figure B

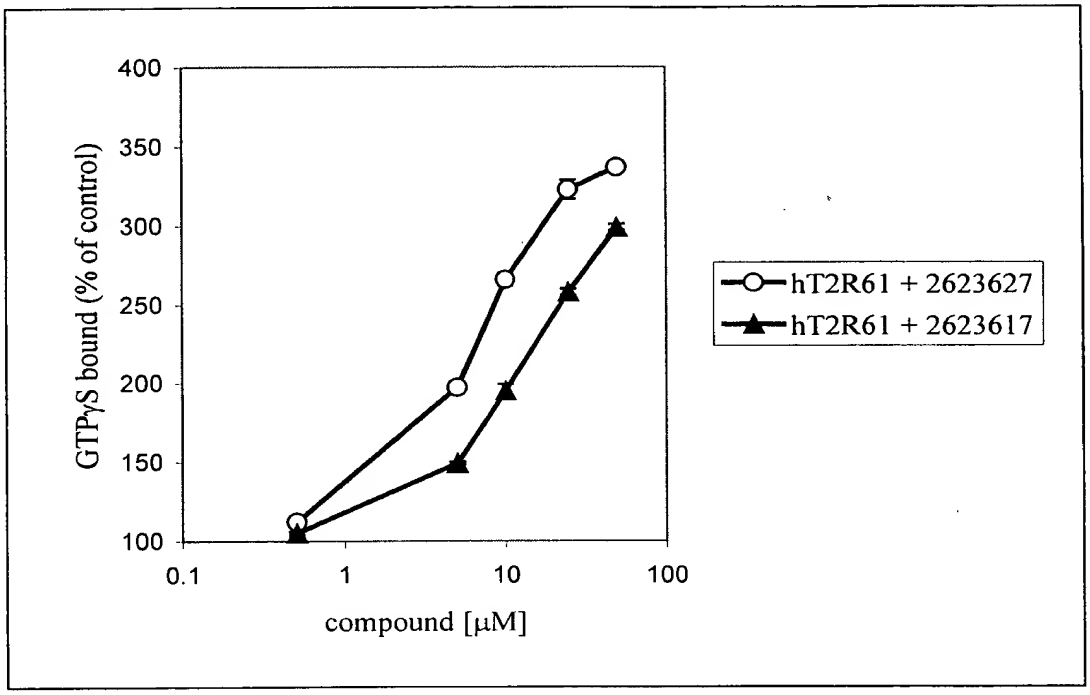

Figure B. Activation of hT2R61 by compounds from 3,4-dihydro-isoquinolin-1-one class. Activity of hT2R61 was determined using GTPγS binding assay either in the absence or presence of indicated concentrations of compounds. The activity is expressed as a percentage of activity in the absence of added test compounds.

Example II: Identification of novel hT2R61 agonists of the benzothiazole class

Figure C contains the results of a GTPγS binding assay that shows that hT2R61 is activated by compounds from benzothiazole class:
#231636 - 2-(Prop-2-ene-1-sulfonyl)-benzothiazole
#2604439 - 2-[2-(4,6-Dimethyl-pyrimidin-2-ylsulfanyl)-ethanesulfonyl]-benzothiazole
#2604447 - 1,2-bis(sulfonylbenzothiazole)ethane

Figure C

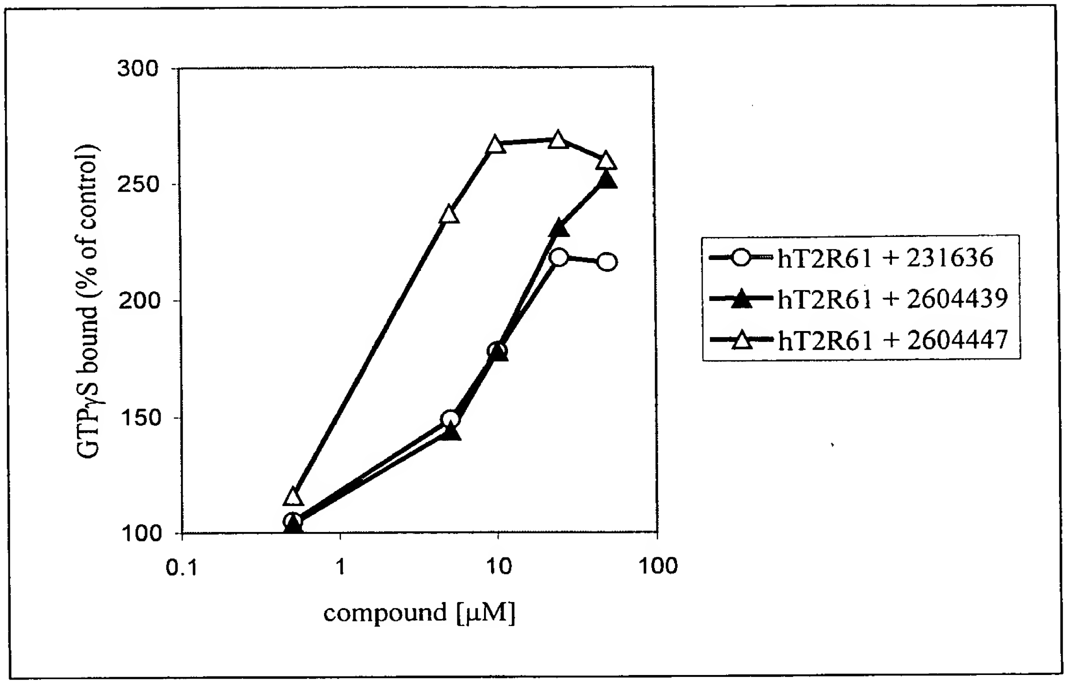

Figure C. Activation of hT2R61 by compounds from benzothiazole class. Activity of hT2R61 was determined using GTPγS binding assay either in the absence or presence of indicated concentrations of compounds. The activity is expressed as a percentage of activity in the absence of added test compounds.

Example IV: Novel agonists of hT2R61 taste bitter to humans

The identified compounds activate hT2R61 at concentrations (5-50 μM) that are significantly lower compared to active 6-nitrosaccharin concentrations (250-2000 μM). To confirm that novel hT2R61 activators are bitter to humans at concentrations that active in GTPγS binding assay, we performed a taste test with 5 human subjects. The most potent compounds from each active chemical class (#2623627 and #2604447) were dissolved in water to achieve final concentration 50 μM. Subsequent analysis demonstrated that actual concentration in solution for #2623627 was 47 μM, whereas for #2604447 it was significantly lower than 50 μM due to poor solubility. Each of the 5 subjects tasted compounds' solution, then described a taste modality and ranked its intensity using labeled-magnitude scale. For comparison, standard solutions of quinine sulfate in water (1.5-40.5 μM) were also tasted and evaluated by each subject. Figure D contains the results of a taste test that shows that hT2R61 activators are bitter to humans.

Figure D

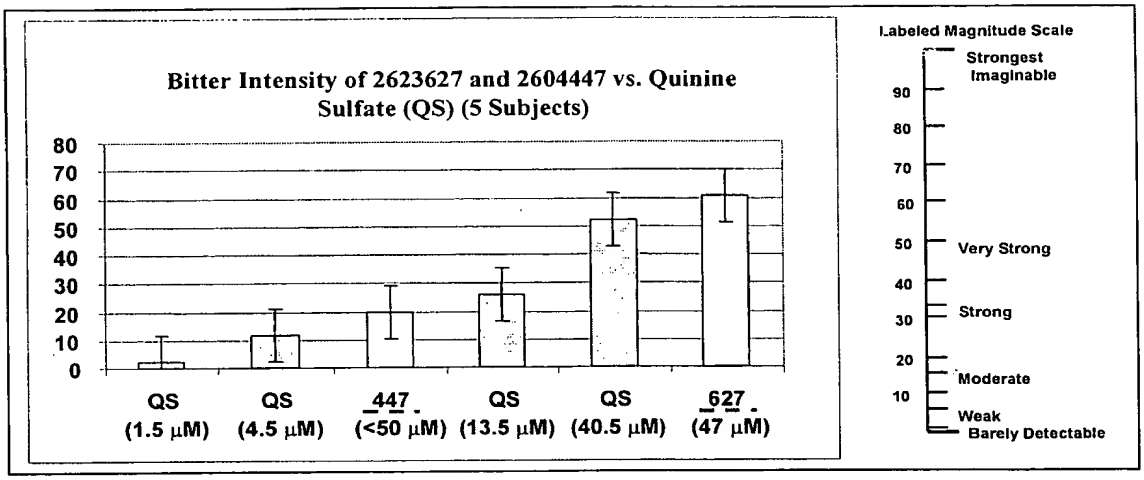

Figure D. Activators of hT2R61 are bitter to humans. Indicated compounds or quinine sulfate were dissolved in water and tasted by 5 human subject. The bitter intensity was ranked from 0 (barely detectable) to 100 (strongest imaginable). The results are represented as the average rating among all 5 subjects.

The main results of the taste test are also summarized in Table 1.

Table 1

Compound	Tested concentration	Bitter taste
quinine sulfate	40.5 μM	strong to very strong
2623627	47 μM	very strong
2604447	<<50 μM	moderate

Summary

These results demonstrate that the GTPγS binding assay for T2R receptors that we invented can be used to identify novel compounds that activate hT2R61. These compounds taste bitter to humans at the concentrations consistent with their activity in the assay. These findings demonstrate the use of the GTPγS binding assay for human T2Rs to identify bitter compounds and demonstrate that hT2R61 is a human bitter receptor for the identified compounds. The compounds described se compounds can be used to provide bitterness to foods and beverages, and can be used as agonists in assays for bitter blockers and modulators. The hT2R61 assay can be used to find additional bitter molecules.

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	: 09/825,882	Confirmation No.	:	3758
Applicant	: JON ADLER			
Filed	: April 5, 2001			
TC/A.U.	: 1646			
Examiner	: M Brannock			
Docket No.	: 100337.54075US			
Customer No.	: 23911			
Title	: T2R TASTE RECEPTORS AND GENES ENCODING SAME			

DECLARATION OF MARK ZOLLER, Ph.D.

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Mark Zoller, Ph.D., declare and state as follows:

(1) That I have been employed by Senomyx, Inc. from March 2000 to the present date. My title is Chief Scientific Officer and Senior Vice President of Research.

(2) That I am an expert in the subject matter claimed in the above-identified application which relates to human G protein coupled receptors (human GPCRs) that are members of a taste receptor family involved in bitter taste sensation and which are referred to in this application and by the relevant scientific community as T2Rs.

(3) That my expertise in the relevant technology is substantiated by my curriculum vitae which is attached as an exhibit to this declaration.

(4) That I am familiar with the file history in the above-identified patent application including the most recent Office Action. Additionally, I attended in February of this year an interview with Examiner Brannock and Supervisory Examiner Eyler wherein the outstanding objections and rejections were discussed. Based thereon, it is my understanding that the

Examiner has suggested that it was not reasonably predictable from the as-filed application and the then-state of the art relating to taste receptors, and specifically T2R members, that the DNA sequence having SEQ ID NO: 7, encoding the polypeptide contained in SEQ ID NO: 8, also referred to as hT2R61, would have been shown to encode a human taste receptor involved in bitter taste sensation. That based on the following, I respectfully disagree:

(5) That as of the date of this invention, a human T2R referred to as human T2R1 and having a sequence related to hT2R61 had been identified in the human genome and found to be linked with bitter taste. (*See, Adler et al.*, "A novel family of mammalian taste receptors"; *Cell* 100(6):611-8 (March 17, 2000)). This discovery provided the first experimental evidence as to the function of T2R members in bitter taste.

(6) That as of the date of invention, the existence of a family of T2Rs comprised in both rodent (rat and mouse) genomes and the human genome had been known (*Adler et al (Id.)*).

(7) That upon the discovery of T2R1, this nucleic acid sequence was used as a probe to identify related T2Rs in rodent and human genomes. In fact, all sequences which were identified using T2R1 encoded GPCRs which are well known to be ligand activated receptors.

(8) That the closest sequence homology of the identified human gene sequences, as well as the T2R sequences disclosed in this application, including hT2R61, were to other T2R members and therefore it was reasonable for the inventors of this application to conclude that these gene sequences encoded GPCRs involved in bitter taste.

(9) That expression data further evidenced that these T2R members were expressed in a subset of taste cells, and were expressed in different cells compared with T1R1 and T1R2-expressing cells (respectively umami and sweet receptors). This result further reasonably suggested to the inventors that T2Rs were responsible for a taste modality distinct from sweet

or umami. Further, we reasonably concluded that the identified T2Rs, including T2R61, were involved in bitter taste, and not salty or sour taste, because the conventional thinking in the taste receptor scientific community at the time of invention and now is that salty and sour taste modalities are regulated by ion channels and not GPCRs.

(10) That the role of T2R members in bitter taste was further supported by experimental data that demonstrated that T2Rs are expressed in cells in association with the G protein gustducin, previously shown by mouse knock out data to be involved in bitter taste. (Wong et al., "Transduction of bitter and sweet taste by gustducin"), *Nature* 381 (658):796-800 (1996)).

(11) That it had been further shown as of the date of invention with other related T2R members that T2Rs are activated by bitter tastants (Chandrashekar et al., *Cell* 100(6):703-11 (March 17, 2000)). Specifically, it was reported by Chandrashekar et al., that mouse T2R5 responded to the bitter ligand cycloheximide and that human T2R4 responds to the bitter ligand denatonium.

(12) That the human T2R members disclosed in this patent application (which were identified in the human genome) were also identified subsequently by another academic lab and were similarly predicted to encode bitter taste receptors (Conte et al., *Cytogenet. Genome Res.* 98(1): 43-53 (2002)). Therefore, there is a consensus in the relevant scientific community that the subject human T2R members, including hT2R61, encode bitter taste receptors. Also, the location and organization of T2Rs in the genome is consistent with gene duplication events leading to gene expression and hence the reasonable expectation that the various T2R members encode functional bitter taste receptors.

(13) That the reasonable expectation as to the functional role of T2R61 in bitter taste is further supported by subsequent ligand data showing that human T2R61 is activated by the bitter ligand nitrosaccharin. Additionally, the function of T2R61 in bitter taste is further evidenced by recent data reported by another research group (Bufe et al.) which showed that human T2R61 (referred to by the Bufe group as human TAS2R44) is activated by the sweetener saccharin, a compound also well known to elicit a bitter taste at elevated concentrations (Bufe et al., "Deorphanization and functional SNP analysis of TAS2R Bitter Taste Receptor", Association of Chemorception Sciences (AChems), Meeting April 21-25, 2004, Abstract #191) In fact, saccharin was among the sweet and bitter ligands mentioned in the Adler et al. (*Id.*) and Chandrashekar et al. (*Id.*) publications incorporated by reference in this application.

(14) That other T2Rs have been shown to respond to bitter ligands as predicted by the inventors of this application. Particularly, T2R16 has been shown to respond to salicin, a bitter ligand, using an assay system described in the above-identified Senomyx patent application (Bufe et al., "The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides", *Nat. Genet.* 32(3):397-401.).

(15) That it is my expert opinion that the above-information considered cumulatively along with the information contained in the above-identified Senomyx patent application supports a conclusion that it was entirely reasonable to anticipate that T2R61 would be shown in bitter ligand functional and binding assays to encode a bitter taste receptor, as correctly disclosed in the Senomyx patent application at issue.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Mark Zoller, Ph.D.
Chief Scientific Officer and Senior Vice President of Research

DATE SIGNED: May 13, 2004

CURRICULUM VITAE

Mark J. Zoller

Personal

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Education

1971-1975	B.A., Chemistry, 1975, Pomona College, Claremont, CA.
1975-1980	Graduate student with Dr. Susan S. Taylor Department of Chemistry, University of California, San Diego M.S., 1977; PhD., 1980

Professional Experience

1981-1983	Post-doctoral research with Dr. Michael Smith, Nobel Laureate Department of Biochemistry, School of Medicine University of British Columbia, Vancouver, B.C.
1983-1988	Senior Staff Investigator Cold Spring Harbor Laboratory, New York
1988-1992	Senior Scientist Department of Protein Engineering Genentech, Inc, South San Francisco, CA.
1989-	Adjunct assistant professor Department of Pharmaceutical Chemistry School of Pharmacy, UCSF, San Francisco, CA
1992-1994	Director, Molecular Biology ARIAD Pharmaceuticals, Cambridge, MA
1994- 1998	Vice President, Drug Discovery - Signal Transduction ARIAD Pharmaceuticals, Cambridge, MA
1998 - 2000	Scientific Director, Hoechst-ARIAD Genomics Center Senior Vice President, Genomics ARIAD Pharmaceuticals, Cambridge,
2000 -	Senior Vice President, Research and Chief Scientific Officer Senomyx, Inc., La Jolla, CA

BIOGRAPHICAL SKETCH -- Mark J. Zoller, Ph.D

Dr. Zoller joined Senomyx in March 2000 as Vice President, Research to work with renown textbook author and scientist, Professor Lubert Stryer, who serves as Senomyx' Chief Scientific Officer. Senomyx is a newly formed biotech company, dedicated to the discovery of novel molecules that modulate taste and olfaction. The company was formed to exploit recent advances in chemosensory genomics for the development of novel flavor and fragrance molecules for consumer products.

Prior to coming to Senomyx, Dr. Zoller held a number of scientific management positions at ARIAD Pharmaceuticals in Cambridge, Massachusetts, most recently as Scientific Director of the Hoechst-ARIAD Genomics Center and Senior Vice President, Genomics, ARIAD Pharmaceuticals. Previously, Dr. Zoller was a Senior Scientist in the Protein Engineering Department at Genentech, where he used protein engineering technologies to create a series of second-generation tissue plasminogen activators (t-PAs). Dr. Zoller's work led to one of these proteins being developed into the recently approved drug, Tenecteplase. Prior to joining Genentech, Dr. Zoller was a Senior Scientist at the Cold Spring Harbor Laboratory. There he did research on protein kinases using yeast molecular genetics, and taught the Advanced Molecular Cloning course from 1983 to 1987.

Dr. Zoller received his Ph.D. in Chemistry in 1980 from the University of California, San Diego working in the laboratory of Dr. Susan Taylor. Dr. Zoller's thesis characterized the structure and function of cAMP-dependent protein kinase. Having trained in a protein chemistry lab, in 1981 Dr. Zoller did post-doctoral research in the laboratory of Nobel Laureate, Professor Michael Smith in Vancouver, British Columbia. There he developed improved methods for oligonucleotide-directed mutagenesis. Since 1989, Dr. Zoller has been an Adjunct Assistant Professor in the Department of Pharmaceutical Chemistry at University of California, San Francisco. He has published over 40 scientific papers, holds 10 issued patents on second generation t-PA, is on the editorial boards of Protein Engineering, and co-authored a molecular biology textbook entitled Recombinant DNA with Nobel Laureate James D. Watson, along with Michael Gilman and Jan Witkowski, Director of the Banbury Center at the Cold Spring Harbor Laboratory. The textbook is widely used undergraduate and graduate molecular biology courses and has sold over 100,000 copies to date.

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